

Exhibit A

Liposome Technology

2nd Edition

Volume II

Entrapment of Drugs and Other Materials

Margit et al.

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Chapter 2

**pH GRADIENT-MEDIATED DRUG
ENTRAPMENT IN LIPOSOMES**

L. D. Mayer, T. D. Madden, M. B. Bally, and P. R. Cullis

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I. INTRODUCTION

Some of the more significant obstacles to be faced in the development of liposome-based pharmaceuticals lie in the ability to produce formulations that not only provide a therapeutic benefit, but also meet pharmaceutical demands such as large-scale production and long-term stability.¹ These issues can be particularly difficult to resolve for many amphiphilic drugs employing passive liposome entrapment procedures, where the drug is included in the hydration buffer during liposome preparation. This is due to the fact that, for passively trapped formulations, the liposomes must retain such drugs (after removal of uncapsulated drug) for 1 year or more during storage, an especially challenging task given the relatively high membrane permeabilities of these dual hydrophobic-hydrophilic agents. Similar problems can be experienced for these systems when cryoprotectant-based storage approaches (freezing and lyophilization) are utilized in that drug leakage can occur at the time of reconstitution.² Furthermore, many agents in various drug classes, such as antineoplastics, are extremely toxic. This presents specific scale-up problems because large-scale liposome processing techniques such as high pressure extrusion must be performed in the presence of the drug, thus making the procedures potentially very dangerous.

The use of transmembrane pH gradients to encapsulate certain amphiphilic drugs resolves many of the problems associated with passive trapping techniques. In this "active entrapment" approach the drugs (lipophilic amines as presented here) are added to preformed liposomes with an imposed pH gradient (inside acidic). The drugs redistribute across the membrane in response to the pH gradient, resulting in trapping efficiencies that can approach 100%.³ This allows the drug to be encapsulated just prior to use, thus alleviating problems associated with postencapsulation processing and long-term storage. In addition, the pH gradient increases the retention of such drugs inside the liposomes once entrapped. Finally, since this approach does not rely on any specific drug-lipid interaction to enhance entrapment, it can be used for virtually any lipid mixture that forms liposomes capable of maintaining a stable transmembrane pH gradient.

The use of transmembrane pH gradients to encapsulate drugs in liposomes is applicable to a wide variety of agents in many drug classes. Table 1 lists several lipophilic, amine drugs that respond to liposomes exhibiting a pH gradient (inside acidic) by accumulating in the vesicle interior. This encapsulation procedure is suitable for drugs ranging from antineoplastics to local anesthetics and antiparasitic agents.⁴ Such information suggests that pH gradient-mediated drug entrapment may be of use in a broad spectrum of applications.

In the sections to follow, the authors describe the techniques for preparing liposomes that exhibit pH gradients, loading the liposomes with drugs and analyzing the resulting systems for drug entrapment, as well as the magnitude

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TABLE I
Lipophilic Amine Drugs That Accumulate Inside Liposomes
in Response to Transmembrane pH Gradients

Drug	Class
Doxorubicin	Antineoplastic
Doxorubicin	Antineoplastic
Epirubicin	Antineoplastic
Mitoxanthrone	Antineoplastic
Vinblastine	Antineoplastic
Vincristine	Antineoplastic
Chlorpromazine	Local anesthetic
Dibucaine	Local anesthetic
Lidocaine	Local anesthetic
Quinidine	Antiarrhythmic agent
Dopamine	Biogenic amine
Serotonin	Biogenic amine
Imipramine	Antidepressant
Diphenhydramine	Antihistamine
Quinine	Antimalarial
Chloroquine	Antimalarial

From Madden, T. D. et al., *Chem. Phys. Lipids*, 53, 37, 1990. With permission.

of the pH gradient. Also discussed is the theory behind the technique and the importance of parameters such as liposome size, lipid composition, drug to lipid ratio, and entrapped buffering capacity in determining the properties of such systems, employing doxorubicin and vincristine as representative drugs.

II. METHODOLOGY

A. PREPARATION OF LIPOSOMES

Volume 1 of this second edition presents a very thorough overview of the various techniques for preparing and evaluating liposomes. In this chapter, issues regarding liposome production that directly influence pH gradient-mediated drug encapsulation are discussed. The liposome preparation techniques described here are meant to serve as examples and the overall concepts should be applicable to most liposome production procedures.

Most liposomes to be used for therapeutic applications are composed of more than one lipid species. It is therefore important to ensure that all the lipid components are homogeneously mixed prior to hydration, regardless of the preparation procedure. Typically, the lipids are co-dissolved in organic solvents such as chloroform, methylene chloride or benzene:methanol (95:5, vol:vol). The solvents can then be removed by high vacuum evaporation in the case of the halogenated solvents or by lyophilization in the case of the benzene:methanol solvent system. The resulting lipid films or lyophilized

powder, respectively, are then ready for hydration in aqueous buffer. It should be noted that for lipid systems containing cholesterol, special attention should be given to the methanol or ethanol content in chloroform during solvent evaporation. During this process, the solvent becomes enriched in alcohol which can result in precipitation of cholesterol in the latter stages of evaporation. Such an occurrence precludes the success of further liposome processing, as the resulting cholesterol microcrystals do not readily incorporate into lipid bilayers after hydration. The remedy for this situation is to avoid the use of alcohol in the preparation of a lipid film from chloroform or methylene chloride solutions and to maintain elevated solution temperatures (40°C) during evaporation. Finally, it is imperative that residual solvent content in the lipid film or powder be negligible, since the presence of solvent in the liposome solution may adversely alter the ability of the liposomes to maintain a stable pH gradient and retain entrapped drug.

Hydration of lipid films or powders can be accomplished by adding buffer of the desired composition and pH and swirling or vortexing until all of the lipid is evenly dispersed in solution (50 to 200 mg lipid per milliliter of buffer). In the case of saturated acyl chain phospholipids, the buffer solution should be maintained above the transition temperature of the highest melting lipid species throughout the hydration process. The resulting multilamellar vesicles (MLVs) are heterogeneous with regard to size (500 nm to several microns in diameter) and display small aqueous trapped volumes ($\leq 1.0 \mu\text{l}/\mu\text{mol lipid}$). In addition, these MLVs often do not have interlamellar equilibrium solute distribution, a property that can give rise to destabilizing transmembrane osmotic gradients.⁵ The trapped volume and solute distribution problems can be alleviated by freezing and thawing the liposome solution several times using liquid nitrogen and a water bath equilibrated above the transition temperature of the highest melting lipid component.⁶ This process results in interlamellar equilibrium solute distribution and increases the aqueous trapped volume of the liposomes above $5 \mu\text{l}/\mu\text{mol lipid}$. Similar increases in trapped volume and equilibrium solute distribution can be accomplished by employing reversed-phase solvent evaporation techniques. However, special attention must be given to ensure complete solvent removal during this procedure in order to avoid the membrane permeability problems referred to above.

Although precursor vesicles such as frozen and thawed MLVs can be used directly to entrap drugs in response to pH gradients, their size heterogeneity is often unacceptable for therapeutic applications. Consequently, additional processing to achieve the desired size distribution is often necessary. Several techniques exist for adjusting liposome size, such as sonication, homogenization, microfluidization, detergent dialysis, and extrusion (see related chapters in Volume 1). The authors have found high pressure extrusion to be the most versatile procedure for their applications since a wide range of mean vesicle diameters can readily be selected on the basis of the filter pore size

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used.⁷ In addition, this procedure does not employ agents such as detergents, which can compromise the drug retention properties of the liposomes.

B. GENERATION OF TRANSMEMBRANE pH GRADIENTS

Selection of the buffer composition and pH during liposome hydration and subsequent processing establishes the conditions of the vesicle interior aqueous compartment. In order to create a transmembrane pH gradient (inside acidic), the pH of the extravesicular aqueous compartment must be increased. This can be accomplished by two general approaches: (1) addition of an alkalinizing agent (typically a concentrated base or alkaline buffer) to increase the exterior pH to the desired level, or (2) exchanging the extravesicular media with the desired buffer by gel exclusion column chromatography or dialysis techniques. For the alkalinizer addition technique, solutions of sodium carbonate or dibasic phosphate (concentrations between 0.1 and 1.0 M) can be used to rapidly adjust the extravesicular pH to the desired level (typically pH 7.0 to 7.5) for conditions under which sterility can be easily maintained. Potential drawbacks are that transmembrane osmotic gradients can result upon addition of the alkalinizer and buffer/drug incompatibilities can limit the choice of the alkalinizing agent. Exchanging the exterior buffer by chromatography or dialysis provides more flexibility in selecting the extravesicular buffer composition, thereby avoiding transmembrane osmotic gradients. However, these techniques are more cumbersome and maintaining sample sterility can be problematic.

C. DRUG ENTRAPMENT

Once the liposomes have been manipulated to exhibit a transmembrane pH gradient they should be used for drug entrapment within a time frame in which the ΔpH has not been significantly depleted. This time will depend largely on the lipid composition and can be determined by monitoring the transmembrane pH gradient as described in the following section. Although it is typically most convenient to alkalinize an aliquot of liposomes with 0.5 M Na_2CO_3 or 0.5 M Na_2HPO_4 for immediate use,^{8,9} we have observed that liposomes displaying a transmembrane pH gradient can be stored unchanged at 4°C for several days.¹⁶ The alkalinized liposomes are then mixed with the appropriate amount of drug dissolved in saline (drug concentration 1.0 to 10.0 mg/ml). Some liposomal preparations will require incubation at elevated temperatures to accomplish complete uptake of the drug into the vesicles. Figure 1 shows the influence of the incubation temperature on doxorubicin uptake into EPC:cholesterol (55:45, mol:mol) liposomes displaying a pH gradient (pH 4.0 inside and pH 7.5 outside). At 37°C, approximately 100% of the drug is entrapped by the liposomes over 90 min. Increasing the temperature to 60°C results in trapping efficiencies approaching 100% within 2 minutes.⁸ For more membrane permeable drugs such as vincristine, incubation temperatures can be lowered, depending on the selection of lipid components.⁹

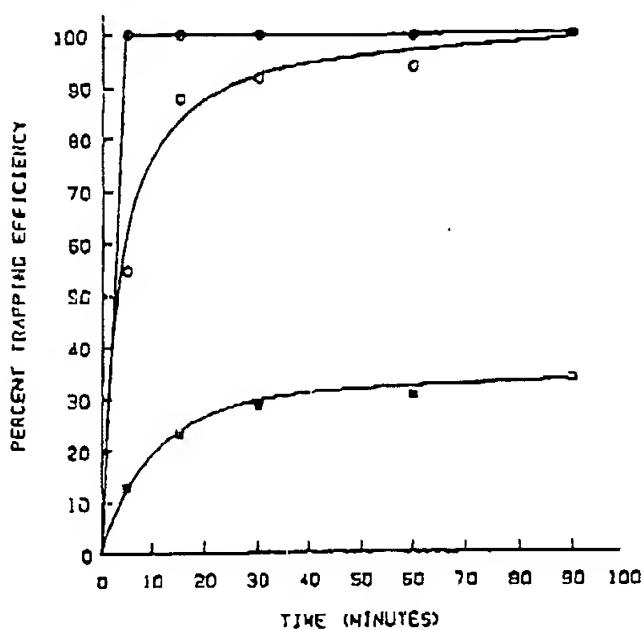


FIGURE 1. Effect of incubation temperature on pH gradient-mediated doxorubicin uptake into EPC:cholesterol (55:45, mol:mol) vesicles. Vesicles were prepared in 300 mM citric acid (pH 4.0) and extruded through 200 nm pore size polycarbonate filters. Prior to doxorubicin addition, the external vesicle medium was brought to pH 7.8 with sodium hydroxide. Doxorubicin (3.0 mg/ml) was added to liposomes (11.0 mg/ml) equilibrated at 21 (■), 37 (○), and 60°C (●). Entrapped doxorubicin was determined as described in the Methodology section. (From Mayer, L. D. et al., *Cancer Res.*, 49, 5922, 1989. With permission.)

Conditions required to achieve $\geq 95\%$ trapping efficiency within a desired length of time should be determined for each drug/liposomal combination used. Once identified, these conditions will yield very reproducible entrapment properties. It should be pointed out that, for some agents, the drug must be added to the liposome solution prior to the alkalinization step to avoid solubility problems. For example, addition of vincristine to DSPC:cholesterol (DSPC, distearoyl phosphatidylcholine) liposomes alkalinized to pH 7.5 results in drug precipitation, whereas alkalinization of the liposomes with 0.5 M Na_2HPO_4 after addition of the drug results in complete drug solubility and efficient encapsulation.

D. DETERMINATION OF DRUG ENTRAPMENT, ΔpH , AND DRUG RETENTION

The extent of drug entrapment by vesicles can be readily determined by column chromatography. A small aliquot of the liposomal drug solution is diluted (if necessary) with unbuffered saline to achieve a lipid concentration of 5 mM. Lipid and drug concentrations are then determined employing scintillation counting, spectrophotometric assays, fluorescence assays, or high

pressure liquid chromatography (HPLC) analysis depending on the nature of the drug and whether the liposomes contain a radiolabeled lipid marker. Untrapped drug is separated from the liposomes by centrifuging 0.1 ml of the diluted sample through a 1.0-ml Sephadex® G-50 minicolumn equilibrated in saline buffered to the pH of the encapsulation solution.⁴ The vesicle-containing eluant fraction is then assayed for lipid and drug content. Trapping efficiencies are calculated as the drug:lipid ratio after separation of free drug divided by the drug:lipid ratio before separation.

Transmembrane pH gradients are monitored employing ¹⁴C-methylamine as a probe of ΔpH . Typically, ¹⁴C-methylamine is added to a liposome solution containing $\leq 10 \text{ mM}$ lipid to achieve a final level of approximately $0.5 \mu\text{Ci}/\text{ml}$. Samples should be incubated for 15 min at a temperature above the transition temperature of the highest melting lipid. After cooling the mixtures to room temperature, 0.1 ml aliquots are passed down 1.0 ml Sephadex® G-50 columns equilibrated at 21°C with a buffer comparable in osmotic strength and pH to the incubation media to remove unencapsulated methylamine. Methylamine and lipid concentrations before and after column chromatography are determined by scintillation counting and lipid phosphorus assay for liposomes that do not contain a radiolabeled marker. Upon determination of the aqueous trapped volume of the vesicles (refer to Chapter 8, Volume I of this set) the transmembrane pH gradient is then calculated according to the relationship:

$$\frac{[\text{H}^+]_{in}}{[\text{H}^+]_{out}} = \frac{[\text{methylamine}]_{in}}{[\text{methylamine}]_{out}}$$

It should be noted that radioactive methylamine of the highest specific activity should be utilized for determinations of transmembrane pH gradients in liposomal systems with very small entrapped buffering capacities and/or very small pH gradients ($< 0.5 \text{ U}$). Methylamine molecules redistribute to the vesicle interior sequester protons in a stoichiometric fashion. As a result, special attention should be given to ensure that conditions used to monitor the transmembrane pH gradient do not, in themselves, cause a reduction of the ΔpH .¹⁹

The *in vitro* retention of liposomal drugs encapsulated in response to pH gradients can be monitored by dialyzing samples (2 mM lipid) for 24 h vs. 1000 vol of 20 mM Hepes, 150 mM NaCl (pH 7.5) at 37°C . At various times 0.15 ml aliquots are removed and entrapped drug is determined employing column chromatography, as described above. The residual pH gradient of these samples can be determined by incubating the aliquot for 15 min with the appropriate amount of methylamine prior to passage down gel filtration columns.

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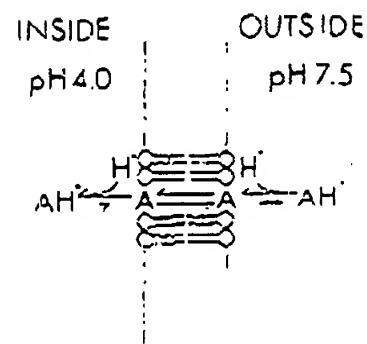


FIGURE 2. Schematic representation of equilibria involved in uptake of lipophilic amine drugs across bilayer membranes in response to transmembrane pH gradients. The neutral (unprotonated) drug species is denoted as "A", whereas the positively charged (protonated) drug species is denoted as "AH⁺".

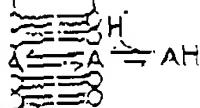
III. RESULTS AND DISCUSSION

A. THEORETICAL CONSIDERATIONS

Lipophilic drugs that contain titratable, amine moieties permeate bilayer membranes orders of magnitude faster in the neutral (uncharged) form than in the positively charged form. As a result, the neutral species of many lipophilic, amine drugs (see Table 1) can readily equilibrate across membranes. As shown in Figure 2, drug molecules on both sides of the membrane will equilibrate between protonated and neutral forms on the basis of the pK_a of the drug and the pH of the bulk media. Assuming that the pK_a of the drug is the same in the intra- and extravesicular aqueous compartments, the relationship between the proton concentration gradient and protonated drug (AH⁺) concentration gradient can be simplified to $[H^+]_{in}/[H^+]_{out} = [AH^+]_{in}/[AH^+]_{out}$. For unilamellar vesicles exhibiting a transmembrane pH gradient of 3 U (interior pH of 4.0 and exterior pH of 7.0), this relationship predicts a drug concentration gradient of 1000 (assuming a drug pK_a that is substantially higher than the pH of the extravesicular medium). One of the first reports of such behavior for biologically active molecules was presented by Nicholls and Dearmer,¹⁰ who demonstrated that dopamine could be accumulated inside model membrane vesicle systems in response to a proton gradient (inside acidic). Numerous studies by the authors' laboratory^{3,4,8,9,11-13} have demonstrated that this response can be utilized to accomplish the efficient entrapment of a wide variety of drugs to high concentrations inside liposomes. The resulting preparations are well suited for therapeutic applications.

Although the analysis presented above suggests that the generation of liposomal drug systems with trapping efficiencies above 90% is straightforward, the ability to obtain stable preparations with specific entrapment, size, and drug:lipid ratio characteristics requires a more thorough examination of the uptake process. Figure 2 shows that protons are consumed as the neutral drug species equilibrates with the charged form upon exposure to the acidic vesicle interior, resulting in a net depletion of the entrapped proton pool. Failure to ensure a sufficient intravesicular buffering capacity can therefore lead to a collapse of the transmembrane pH gradient and a preparation with inferior encapsulation and drug retention properties. This effect will depend

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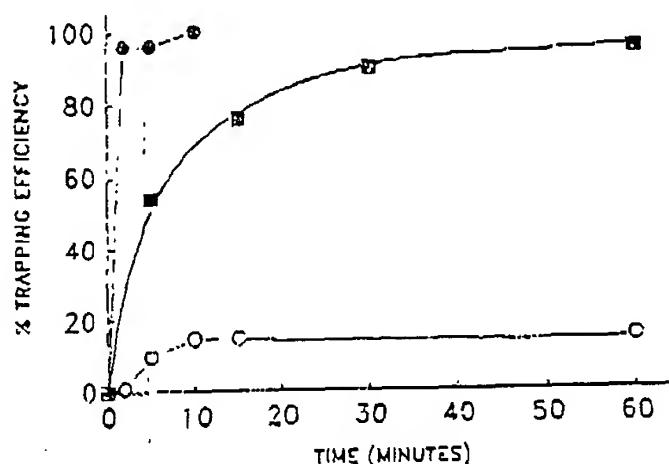


FIGURE 3. Uptake of vincristine into DSPC:cholesterol (●, ○) and EPC:cholesterol (■) vesicles at 21°C (■, ○) and 60°C (●). Vincristine was incubated with PC:cholesterol vesicles (55:45, mol:mol) at a drug:lipid ratio of 0.17:1 (w/w) and trapping efficiencies were determined as described in the Methodology section. (From Mayer, L. D. et al., *Cancer Res.*, 50, 575, 1990. With permission.)

on the vesicle size, drug:lipid ratio, and the number of protonatable groups per drug molecule. Consequently, these parameters must be carefully analyzed for individual liposomal drug formulations prepared by the pH gradient-mediated drug entrapment procedure. Further, the trapping efficiency is related to the absolute drug and lipid concentrations. Specifically, decreasing the liposome concentration for a given drug:lipid ratio will tend to result in decreased drug trapping efficiencies. In the sections to follow, these relationships are described in more detail and are related to specific liposomal doxorubicin and vincristine formulations.

B. INFLUENCE OF LIPID COMPOSITION

Alterations in the lipid composition of liposomes for use in pH gradient-mediated drug encapsulation may be made on the basis of biological response requirements or pharmaceutical issues such as chemical and physical stability. These changes often involve manipulations of cholesterol content or the degree of saturation of the phospholipid acyl chains. In the context of pH gradient-mediated liposomal drug entrapment, changes in lipid composition primarily affect the relative rates of drug uptake and drug retention, whereas characteristics such as the maximum attainable drug:lipid ratio and pH gradient depletion are influenced to a far lesser extent.

Figure 3 shows the dependence of the rate of vincristine uptake into liposomes on lipid composition and incubation temperature. At an incubation temperature of 21°C vincristine accumulates inside EPC/cholesterol (55:45, mol:mol) liposomes, achieving 78% drug entrapment within 15 min and >90% entrapment within 1 h. In contrast, DSPC:cholesterol (55:45, mol:mol)

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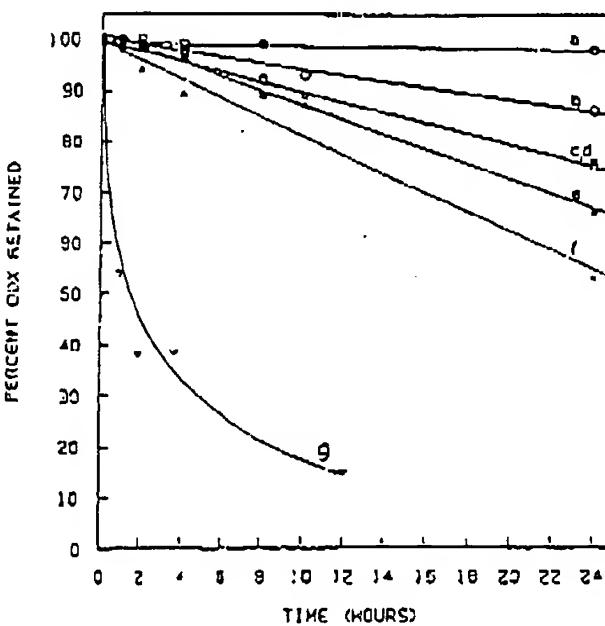


FIGURE 4. Release of doxorubicin from liposomes under dialysis conditions at 37°C after pH gradient-mediated encapsulation (a to f) or passive encapsulation (g). The liposomes were composed of EPC:cholesterol at molar ratios of 55:45 (a, g), 67:33 (b), and 85:15 (c); pure EPC (f); EPC:EPG:cholesterol at molar ratios of 52.5:2.5:45 (c) and 27.5:27.5:45 (d).

liposomes entrap only 20% of the available vincristine over 1 h at this temperature. However, the DSPC:cholesterol liposomes are capable of encapsulating high levels of vincristine, since elevation of the incubation temperature to 60°C results in rapid and efficient ($\geq 95\%$) vincristine entrapment for this vesicle system. Note that the relatively stable uptake level observed for the DSPC:cholesterol liposomal system between 15 and 60 min suggests that this effect is not related solely to permeability rates. pH gradient-mediated uptake properties not only vary as a function of lipid composition, but also according to the drug to be entrapped. For example, whereas vincristine accumulates relatively rapidly in EPC:cholesterol liposomes at 21°C (Figure 3), doxorubicin incubated with these liposomes under identical temperature conditions results in only 30% entrapment over the same time course (Figure 1).

In addition to influencing the pH gradient-dependent uptake process, vesicle lipid composition can also exert effects on the drug retention properties of the liposomes subsequent to encapsulation. As shown in Figure 4, liposomal doxorubicin preparations varying only in lipid composition exhibit different drug release kinetics at 37°C. Three general trends can be identified from these data and other similar studies. First, increasing the cholesterol content of the bilayer membrane increases the ability of the liposomes to retain entrapped doxorubicin. Second, increasing the degree of acyl chain saturation of the phospholipid component (which increases membrane rigidity) increases

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the drug retention properties of the liposomes. Third, inclusion of negatively charged lipids in the membrane results in increased leakage of lipophilic cations such as doxorubicin (Figure 4) and vincristine²⁰ from liposomes after pH gradient-mediated drug encapsulation.

C. INFLUENCE OF ENTRAPPED BUFFER COMPOSITION

As noted above, it is generally accepted that lipophilic amines permeate membranes in the neutral (deprotonated) form. Thus, vesicle uptake of these agents in response to transmembrane pH gradients results in a depletion of the internal proton pool as the neutral species is reprotoxinated upon exposure to the acidic intravesicular medium. Consequently, pH gradient-mediated drug encapsulation is dependent on the entrapped buffering capacity and a major determinant of this parameter is buffer composition. The three primary factors in this area are buffer chemical composition, buffer concentration, and the preuptake pH relative to the pK_a of the selected buffer.

For liposomal systems exhibiting transmembrane pH gradients (inside acidic) in which the internal pH is <5.0 we have found citric acid to be the buffer of choice. It has several advantages over other buffers that are particularly applicable for drug delivery applications. First, it is a widely used, pharmaceutically acceptable excipient for use in injectable therapeutic formulations. Limitations must be placed on the total amount and concentration of citrate administered *in vivo*, however, due to its chelation of plasma calcium. Second, citric acid is a triprotic buffer that exhibits a wide buffering range (pK_a 3.0 to 6.5). Third, because citric acid has three titratable groups, osmotic contributions on a per proton equivalent basis are reduced as compared to diprotic and monoprotic buffers such as oxalic acid and acetic acid. This is an important consideration since liposomal systems exhibiting hyperosmolar intravesicular media are susceptible to serum- and plasma-induced leakage of entrapped contents.

Figure 5 demonstrates that for the lipophilic amine drug doxorubicin pH gradient-mediated drug entrapment is dependent on the buffer concentration in the entrapped aqueous compartment.¹³ For an initial doxorubicin to lipid ratio of 0.36:1 (mol:mol), increasing the citrate concentration from 10 to 100 mM produces an increase in the doxorubicin trapping efficiency from 24% to >98%. Further increases in the entrapped citrate concentration above 100 mM result in trapping efficiencies of approximately 100%. Again, this is consistent with the consumption of entrapped protons during the uptake process, where citrate concentrations below 100 mM result in a collapse of the transmembrane pH gradient and inhibition of further drug uptake. The relationship between drug uptake and buffer concentration will also depend on the vesicle aqueous captured volume and the number of titratable groups per drug molecule.

The buffering capacity inside the liposomes also can be increased by lowering the pH of the intravesicular media. The authors have observed that

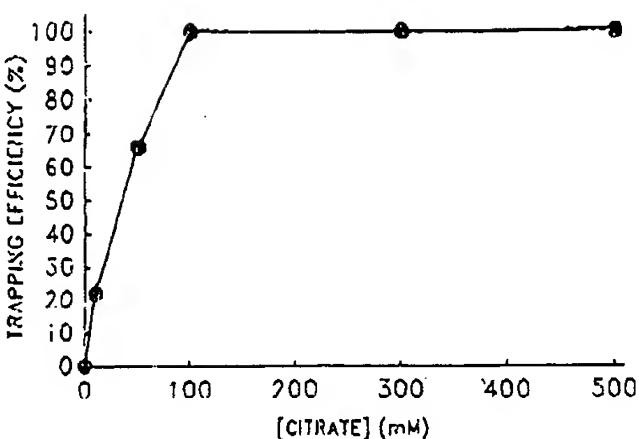


FIGURE 5. Effect of internal buffering capacity on doxorubicin uptake into EPC:cholesterol liposomes extruded through 200 nm pore size polycarbonate filters. Vesicles were prepared in the presence of the indicated citric acid concentrations (pH 4.0). Vesicles (15 mM lipid) were incubated in the presence of doxorubicin (5 mM) for 5 min at 60°C after the vesicle external medium was adjusted to pH 7.8. Trapping efficiencies were determined as described in the Methodology section. (From Mayer, L. D. et al., *Biochim. Biophys. Acta*, 1025, 143, 1990. With permission.)

decreasing the pH of the citrate buffer below 4.0 results in the ability to increase the amount of doxorubicin that can be accumulated for a given citrate concentration. In addition, the pH gradient remaining subsequent to the uptake process is increased. However, a drawback to this approach is that further decreases in the pH exacerbates lipid stability problems associated with acid catalyzed hydrolysis of acyl chains in the phospholipid components.

D. INFLUENCE OF THE DRUG:LIPID RATIO

Given the relationship between drug uptake and depletion of the entrapped proton pool, it may be expected that attempts to encapsulate extremely high levels of drug will result in a collapse of the transmembrane pH gradient and reduced drug trapping efficiencies. The maximum drug uptake obtainable while maintaining a significant postencapsulation pH gradient can be determined by monitoring trapping efficiency, liposomal drug uptake, and ΔpH as a function of the drug:lipid ratio in the incubation mixture.¹³ Figure 6 presents such an analysis for doxorubicin uptake into 175 nm EPC:cholesterol (55:45, mol:mol) liposomes. Varying the drug:lipid ratios for vesicles containing 300 mM citrate (pH 4.0) between 0.11:1 and 0.36:1 (mol:mol) has no effect on doxorubicin trapping efficiency and values of approximately 100% are achieved in this range. Increasing the incubation drug:lipid molar ratio above 0.36:1 results in uptake levels as high as 1.2 mol/mol lipid (Figure 6B). It should be noted that such entrapped drug:lipid ratios are several-fold higher than those obtainable by traditional passive entrapment techniques. Trapping efficiencies decrease significantly, however, as the initial drug:lipid

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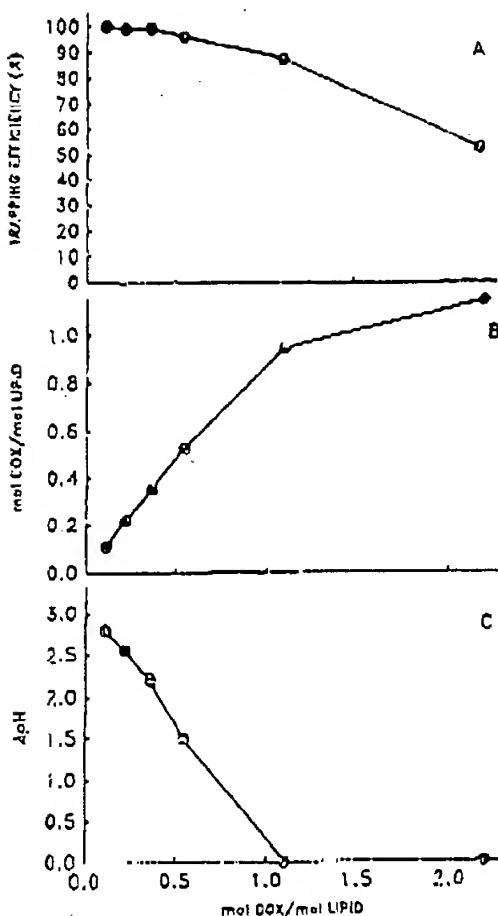


FIGURE 6. Effect of the drug to lipid ratio on doxorubicin trapping efficiency (A), entrapped drug to lipid ratio (B), and post-encapsulation pH gradient (C). EPC/cholesterol 175 nm vesicles (500 mM citric acid, pH 4.0 inside; pH 7.8 outside) were incubated (5 mM lipid) at 60°C for 5 min in the presence of doxorubicin at the indicated drug to lipid ratios. The amounts of entrapped drug as well as trapping efficiencies were determined as described in the Methodology section. Transmembrane pH gradients were determined subsequent to doxorubicin uptake by monitoring the transmembrane distribution of radioactive methylamine and correlating inside/outside concentrations to the transmembrane proton gradient as described in the Methodology section. (From Mayer, L. D. et al., *Biochim. Biophys. Acta*, 1025, 143, 1990. With permission.)

ratio is increased above 0.55:1 (mol:mol). The reason for decreased trapping efficiencies observed for the high drug:lipid ratios can be seen in Figure 6C, in which the transmembrane pH gradient remaining after encapsulation is monitored. Systems exhibiting incubation drug:lipid ratios below 0.36:1 (mol:mol) maintain pH gradients in excess of 2.0. Drug entrapment does deplete the internal proton pool for all systems studied such that ΔpH values are lower than initially imposed. This effect becomes most pronounced for initial drug:lipid ratios $>0.55:1$ (mol:mol) where the postencapsulation pH gradient falls below 1.5 and corresponding trapping efficiencies decrease below 90%.

E. INFLUENCE OF VESICLE SIZE

Several reports^{8,14-16} indicate that decreasing vesicle size may be advantageous for a wide variety of therapeutic liposome applications. Studies have demonstrated that small liposomes are able to accumulate in disease sites such as tumors and exhibit extended circulation lifetimes,¹⁵⁻¹⁷ a property desirable for designing slow release drug delivery carriers. Since decreasing the vesicle size will reduce the entrapped buffering capacity of the liposomes due to the diminished aqueous captured volume, it is important to characterize the relationship between liposome size and drug trapping efficiency as well as pH gradient stability.

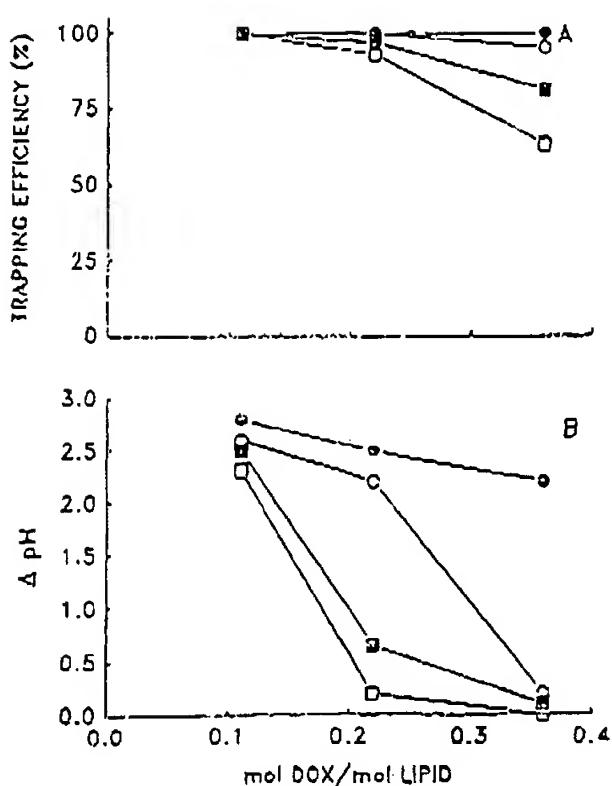


FIGURE 7. Percent doxorubicin trapping efficiency and postencapsulation pH gradient for EPC/cholesterol (55:45, mol:mol) vesicles obtained by extrusion through 200 (●), 100 (○), 50 (■), and 30 nm (□) pore size polycarbonate filters. The mean diameters of these systems were 175, 98, 65, and 55 nm, respectively. All vesicles were prepared in the presence of 300 mM citrate buffer (pH 4.0). The indicated drug to lipid ratios reflect initial drug to lipid ratios in the incubation mixture (lipid concentration, 5 mM). Percent trapping efficiencies and postencapsulation pH gradients were determined as described in the Methodology section. (From Mayer, L. D. et al., *Biochim. Biophys. Acta*, 1025, 143, 1990. With permission.)

Figure 7 shows the dependence of doxorubicin trapping efficiency and residual pH gradient on vesicle size for EPC/cholesterol (55:45, mol:mol) liposomes as a function of the incubation drug:lipid ratio.¹³ The vesicles were extruded through 200, 100, 50, and 30 nm pore size polycarbonate filters, resulting in measured mean diameters of 175, 98, 65, and 55 nm, respectively. Decreasing the vesicle size from 175 to 55 nm decreases the doxorubicin trapping efficiency from approximately 100 to 66%. A more dramatic difference is revealed in the postencapsulation pH gradient in which a value of 2.2 is observed for the 175 nm system while vesicles exhibiting a mean diameter of <100 nm retained a pH gradient of <0.5 U. At a drug:lipid ratio of 0.22:1 (mol:mol), trapping efficiencies in excess of 90% are obtained for all vesicle systems studied; however, residual pH gradients decrease with decreasing liposome size. Decreasing the drug:lipid ratio further to 0.1:1 (mol:mol) yields liposomal doxorubicin preparations that display trapping efficiencies in excess of 98% and postencapsulation pH gradients in excess

of 2.0 U. For liposomes with mean diameters <100 nm, increased trapping efficiencies and residual pH gradients can be achieved by increasing the entrapped citrate concentration above 300 mM (data not shown).

F. DRUG-SPECIFIC EFFECTS

The discussions to this point have assumed ideal behavior for drug redistribution across bilayer membranes in response to transmembrane pH gradients. However, for many drugs (including doxorubicin) the uptake levels achieved indicate transmembrane drug concentration gradients that are in excess of the pH gradient.¹³ Although such deviations from ideality are not fully understood, two parameters are likely to affect the final uptake characteristics of pH gradient-mediated drug entrapment. First, most drugs that can be entrapped inside liposomes in response to pH gradients have substantial lipophilic character. As a result, these agents will display a capacity to associate with or partition into the lipid bilayer. Due to the relatively high membrane surface area:aqueous volume ratio of the vesicle interior, it may be expected that a significant portion of accumulated drug molecules will partition into the membrane. The extent to which this influences pH gradient-dependent drug uptake will depend largely on the lipophilicity of the drug in question, the pH of the vesicle interior, and the maximum capacity of the membrane for drug insertion. If the membrane-bound drug fraction is significant relative to the intravesicular soluble drug fraction, total uptake levels may be expected to surpass those predicted by the Henderson-Hasselbach relationship. Second, many of the systems described here display very high entrapped drug:lipid ratios that represent apparent intravesicular drug concentrations above 100 mM. As shown in Table 2, many lipophilic, cationic drugs have maximum solubilities far below this concentration.⁴ Consequently, it is unlikely that these accumulation levels represent actual drug concentrations that can be related directly to the transmembrane proton concentration gradient. The ability of these drugs to form microprecipitates or alternate phases inside liposomes would also increase equilibrium uptake levels since the exterior drug concentration would need to be depleted until $[\text{solubleAH}^+]/[\text{solubleAH}^+]_{\text{ext}} = [\text{H}^+]_{\text{int}}/[\text{H}^+]_{\text{ext}}$. Again, the relative importance of these phenomena has not been fully defined, however, such properties may be useful in enhancing drug encapsulation characteristics.

IV. CONCLUDING REMARKS

The use of transmembrane pH gradients to encapsulate drugs in liposomes represents a versatile technique to achieve well-defined formulations for a variety of therapeutic agents and liposome types. By monitoring drug entrapment, drug retention in the liposomes, and the transmembrane pH gradient, liposomal formulations can be designed to exhibit specific lipid compositions,

TABLE 2
Apparent Maximum Drug Solubility
in 300 mM Citrate (pH 5.0)

Drug	Apparent max solubility (mM)
Mitoxantrone	<0.01
Epirubicin	0.26
Daunorubicin	9.10
Doxorubicin	0.24
Vincristine	>55
Vindesine	19.1
Lidocaine	240
Dihucine	>700
Propranolol	326
Timolol	135
Quinidine	5.83
Dopamine	1400
Imipramine	4.43
Quinine	1.05
Chloroquine	585
Quinacrine	90

From Madden, T. D. et al., *Chem. Phys. Lipids*, 53, 37, 1990. With permission.

size distributions, and drug:lipid ratios while maintaining high trapping efficiencies and minimal drug leakage. Several features of this process are particularly desirable for pharmaceutical applications. First, the ability to entrap virtually 100% of the drug in preformed liposomes enables the formulation to be constituted just prior to use. This alleviates possible stability problems related to drug chemical stability and drug retention in the liposomes that are associated with traditional passive entrapment procedures. In addition, manufacturing concerns are greatly simplified by the fact that highly toxic drugs are not present during liposome manufacturing processes. Second, the pH gradient imparts excellent drug retention properties to the liposomal systems subsequent to drug uptake. This property is particularly germane for applications in which liposomes are utilized as slow release drug carriers in the circulation. Third, since drug uptake is relatively independent of vesicle lipid composition, potentially labile lipids can be omitted unless dictated by biological response requirements. Fourth, drug:lipid ratios far greater than those previously obtainable can be achieved, thus reducing production costs associated with lipid components. These properties suggest that pH gradient-mediated drug encapsulation will be well suited for preparing therapeutically effective and pharmaceutically acceptable liposomal formulations for a wide range of drugs.

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Water Permeability of Lipid Membranes

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I. INTRODUCTION

Water balance is a central feature in the adaptation of an animal to its environment. Any discussion of water balance requires consideration of the movement of water across cells. The swelling or shrinking of a cell when placed in a nonisotonic solution is usually taken to indicate that the cell contents are enclosed in a semipermeable membrane (88). This cell membrane behaves as a selective permeability barrier to the passage of polar solutes and water into and out of the cell and is thought to consist to some extent at least of a bimolecular lipid leaflet. In practice a quantitative determination of the water permeability of a cell membrane is often complicated by the presence of other diffusion barriers and by the geometrical complexity of the tissue. The study of water transport in artificial lipid membranes, the subject of this review, may help to improve our understanding of the problems in the interpretation of measurements on biological membranes. In addition, while bearing some similarities to biological membranes, the artificial system is sufficiently simple chemically for there to be some prospect of understanding the mechanisms of water transport. Moreover these mechanisms may also apply to the more complex biological membranes.

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Two methods have been used to measure the water permeability of the cell membrane. The diffusional permeability coefficient (P_d) has been determined by measuring the exchange of radioactively labeled water between the cell and its surroundings. Alternatively a permeability coefficient (P_r) has been obtained from the hydraulic conductivity of the membrane, the latter being inferred from the net water flux generated by a given gradient of an osmotic or, more rarely, a hydrostatic pressure. In practice this usually involves measuring the rate of change of the volume of a cell when it is immersed in a nonisotonic solution. Both diffusional and net flux techniques have been applied most extensively to free-living cells such as erythrocytes (51, 97, 130) or eggs (81, 87, 88, 106). Even for such simple single-cell systems, there have been difficulties in interpreting the results. These revolve around whether the cell membrane really constitutes the rate-limiting step in the transport of water and solutes. It has been argued that the diffusion of water both within the cytoplasm (34) and in unstirred layers outside the cell (32) may pose extra limitations to transport. The unstirred layer is a layer of static fluid close to the membrane where the concentrations are different from those in the bulk solution. Within such layers there is no convective mixing and movement takes place solely by diffusion. Whether diffusion in these layers limits the rate of movement of a given substance depends largely on the membrane's permeability to that substance, as discussed later.

The presence of unstirred layers can lead to an underestimate in the true membrane permeability. This problem is likely to be most serious for measurements of the diffusional permeability P_d (see sect. II) and could lead to a difference in the apparent permeability determined by the two different methods. To a lesser extent, it will also cause the osmotic permeability P_r to be underestimated. It has been a consistent finding in experiments on biological membranes that P_d was smaller than P_r (72, 97, 106). This was originally interpreted as being due to the fact that water was moving through aqueous channels or pores in the membrane (80, 97, 106). Depending on their dimensions, these channels could present a different resistance to bulk flow and diffusion, and hence the difference in permeabilities was used to try to derive the dimensions of the channel (80, 97, 102, 132). The effect of the presence of both aqueous channels and unstirred layers on the measured permeability ratio P_r/P_d for artificial membranes is discussed later.

Even if in a given system the measurements of the cell water permeability accurately reflect the permeability of the surface membrane, there are still difficulties in understanding the mechanism of transport in such a membrane. The problem arises from the chemical complexity of a biological membrane and the inability to manipulate the composition to any great extent. The lipid bilayer is thought to be a fundamental component of biological membranes, but there is a mixture of lipids present with different head groups and chain compositions, and the permeability and fluidity of the bilayer may also be modified by membrane proteins. The heterogeneity and complexity of biological membranes thus require the use of a simpler system in order to understand some of the detailed mechanisms. The bimolecular lipid leaflet, as a likely component in the biological

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membrane structure, is such a system and is the only type of artificial membrane considered in this review. Water permeabilities have also been measured for artificial membranes formed from other substances [e.g., cellophane, cellulose acetate, Visking dialysis tubing (87, 112, 135)], but these are not discussed here.

Ideally the water permeability of a lipid bilayer should be measured for a single pure lipid leaflet extended across a hole in a septum separating two semi-infinite compartments. The Montal-Mueller "solventless" bilayers (93) seem at first sight to meet these requirements, but in practice their use for water permeability measurements is not technically easy and no work on this system has yet been reported. Two other preparations, the optically black lipid film and the liposomal or vesicular suspension, are available, however, and each has its advantages and disadvantages. These are discussed in detail in sections II and III.

One of the main findings from studying artificial lipid membranes is that the magnitude of the water permeability can vary over nearly two orders of magnitude depending on the type of lipid used. In addition the permeability can be increased further by the addition of polypeptides that form hydrophilic channels through the nonpolar core of the membrane. The conclusions about the mechanisms of water transport across lipid membranes are presented in section IV, along with the significance of such conclusions for biological membranes. This review is not concerned with the detailed results of water permeability measurements on biological membranes and for this kind of information the reader is referred to other sources (85, 51, 72).

II. OPTICALLY BLACK LIPID FILMS

A. Structure and Relation to Lipid Bilayer.

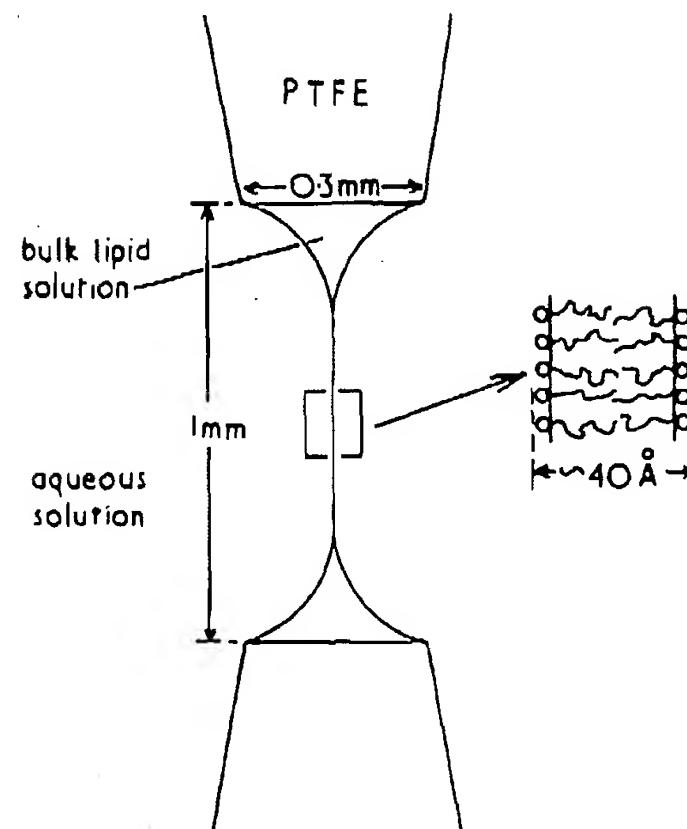
Before presenting the results of water permeability studies on black lipid films, it is desirable to say something concerning their structure and relation to the kind of lipid leaflet likely to be present in biological membranes. Many questions discussed in this section have been covered in more detail in a review of black-film techniques (47).

A "black" lipid film is formed by extending a solution of a lipid in a nonpolar solvent across a hole in a suitable support, under an aqueous solution. On completion of the drainage process the structure is schematically as illustrated in Figure 1. In addition to the planar region of thin membrane in the center of the hole, there is a ring of bulk lipid solution, often described as the annulus but completely analogous to the Plateau-Gibbs border of an aqueous soap film. By an appropriate choice of lipid it is possible to produce a system in which the thin film, the annulus, and the aqueous solution are in chemical equilibrium. From consideration of such a system it is obvious that since the chemical potentials of all components are equal in all phases, there must be some, however little, nonpolar solvent in the membrane. Owing to the presence of this solvent, the black film is never quite comparable to the lipid leaflet found in biological

type of artificial membrane have also been measured for s [e.g., cellophane, cellulose these are not discussed here. er should be measured for a n a septum separating two "solventless" bilayers (93) n practice their use for water and no work on this system optically black lipid film and e, however, and each has its n detail in sections II and III. l lipid membranes is that the over nearly two orders of ddition the permeability can ides that form hydrophilic e. The conclusions about the nes are presented in section biological membranes. This ults of water permeability his kind of information the

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FIG. 1. Cross section through black lipid film in aqueous solution; an annulus of bulk lipid solution connects bilayer region to PTFE support. Dimensions of the bilayer are approximately those for glycerol monooleate/hexadecane films.



membranes. However, as will be shown, solvents of more than a certain molecular volume are largely excluded from lipid bilayers and may occupy only a few percent by volume of the membrane. For systems at equilibrium¹ the presence of the annulus does not necessarily have to be considered when discussing the solvent content of the membrane because the latter is determined by the concentration (or, strictly, the activity) of the solvent in solution in the aqueous phase. Thus, hypothetically at least, a black film of composition identical to the equilibrium films under discussion may be formed by placing a planar lipid leaflet in an aqueous solution of the solvent such that, after adsorption of the solvent, the concentration is similar to that in the actual black-film system. By extension of this line of thought, a biological cell membrane, such as a red blood cell ghost, when placed in an appropriately concentrated solution of nonpolar solvent, will become effectively a spherical black film in which the membrane proteins are embedded.

The clearest examples of the equilibrium black films are probably those involving monoglycerides and *n*-alkanes (7, 46, 115). By contrast most of the phospholipid black films described in the literature exhibit characteristics that are time dependent in some respect (23, 46, 58, 147). With sufficient care during the experimental procedure, however, it is possible to prepare membranes with

¹ Black films, including spherical black films (99), usually have finite tensions, which cause them to collapse when perturbed beyond some critical level. Thus the films are really in a metastable equilibrium. This does not, however, affect the discussion of film structure that follows..

reproducible, stable capacitances (46, 58, 115) that respond reversibly to compression by electric fields (115). These films can remain for hours without significant changes (58) and their structure can thus remain effectively constant over the time of a water permeability experiment.

Black-film structure and composition have been determined by various techniques, but usually a common line of argument is used that briefly is as follows. First, the thickness of the hydrocarbon-chain region of the film is estimated; this yields the volume of unit area of the nonpolar part of the leaflet. Next, the number of lipid molecules per unit area is determined; from this and an approximate knowledge of the molecular volume or density of the lipid chains, the volume occupied by the lipid can be calculated. The difference between this volume and the total volume of the nonpolar part of the membrane is assumed to be occupied by the solvent.

The thickness of the hydrocarbon-chain region of the film may be obtained from electrical capacitance measurements, provided a dielectric constant can be assumed. In fact when alkanes are used as solvents for black-film formation there is little scope for error in this assumption (for a detailed discussion see 47) and the thicknesses obtained are probably accurate to within 2-3 Å.

The number of lipid molecules per unit area has been determined for a few black films, either from the application of surface thermodynamics (29, 47), or by the method of mercury-droplet sampling (47, 98). No serious discrepancies between these two techniques have emerged, and when *n*-alkanes are used as solvents the area per molecule for a given lipid is effectively independent of the alkane selected (7, 46, 98, 145). The film thickness, on the other hand, is strongly dependent on the chain length of the alkane. Tables 1 and 2 show some of the structural features of the better characterized black films. Capacity measurements for the "solventless" membranes² made by the Montal-Mueller method give hydrocarbon-layer thicknesses very close to that obtained from X-ray diffraction in the multilamellar liquid crystal (15, 44, 45, 62). This helps to justify the calculation of thicknesses from capacity measurements. It is also physically reasonable that the smaller alkanes should thicken the membranes more than the larger ones (which are eventually excluded almost entirely) and that the maximum thicknesses found for both the phospholipids and the monoglycerides are about twice the extended chain length of the lipids. A film thicker than this would have a layer of pure solvent in the middle and hence no obvious means of achieving stability.

When cholesterol, in addition to phospholipid, is added to the film-forming solution, the final membranes obtained are usually thinner than in the absence of cholesterol [though not when hexadecane is used as a solvent (46)]. As shown in Table 2 and in a previous publication (63), the introduction of cholesterol

² It is doubtful whether these membranes are ever really solventless, since attempts to form them in the complete absence of any solvent or grease, in our experience, fail. Indeed, theoretical arguments have been advanced that this should be so (146). Nevertheless the Montal-Mueller membranes should contain very little solvent.

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TABLE 1. *Structural features of monoolein black films
and "solventless" bilayers*

Membrane (Monoolein, ca. 5 mM in <i>n</i> -Alkane)	Film Electrical Capacity per Unit Area, μ F/cm ²	Hydrocarbon- Layer Thickness, Å	Volume Fraction of Alkane in Film	Area per Molecule of Monoolein, Å ² /molecule	Ref.
<i>n</i> -Heptane	0.389	47.1	0.48*	39	113
<i>n</i> -Octane	0.385	47.8	0.49	39	
<i>n</i> -Decane	0.386	48.1	0.49*	39	
<i>n</i> -Dodecane	0.414	45.3	0.46	39	
<i>n</i> -Tetradecane	0.465	40.7	0.40*	39	
<i>n</i> -Pentadecane	0.520	36.6	0.38	39	
<i>n</i> -Hexadecane	0.585	32.7	0.26*	39	
None	0.745	25.0	0		15

* These values differ slightly from those in Ref. 46 because different assumptions were made concerning molecular volumes.

substantially reduces the thickness of a lecithin/decane black film and, from the values tentatively deduced for the areas per molecule (29), it can be concluded that a considerable proportion of the decane is probably displaced, giving an almost solventless film.

TABLE 2. *Structural features of phosphatidylcholine black films
and "solventless" bilayers*

Membrane	Film Electrical Capacity per Unit Area, μ F/cm ²	Hydrocarbon- Layer Thickness, Å	Volume Fraction of Alkane in Film	Area per Molecule of Lipid, Å ²	Ref.
Lipid	Solvent				
1,2-Dioleylphosphatidyl- choline	<i>n</i> -Decane	0.390	48.3	0.85	61 116
Egg phosphatidylcholine	Isooctane	0.387	48.2	0.87	61 44
	<i>n</i> -Decane	0.387	48.6	0.37*	61 44, 46
	<i>n</i> -Dodecane	0.445	42.7	0.29*	61 44, 46
	<i>n</i> -Tetradecane	0.526	36.6	0.17*	61 44, 46
	<i>n</i> -Hexadecane	0.603	32.2	0.05*	61 44, 46
	<i>n</i> -Octadecane†	0.627	31.0	0.02	61 44
1,2-Dioleylphosphatidyl- choline	None	0.721	25.8	Zero	15
Egg phosphatidylcholine	None	0.76	25.6	Zero	45
Liquid crystal	None		26‡	Zero	71.7 131
Egg phosphatidylcholine + cholesterol (9 and 28 mM, re- spectively)	<i>n</i> -Decane	0.600	38.6	Small	45

* These values differ slightly from those of Ref. 46 because different assumptions were made concerning molecular volumes. † 22°C. ‡ X ray.

Tables 1 and 2 show that the choice of solvent is important when working with black films. However, the approach described above gives only indirectly the proportion of solvent in the film and it would be useful to be able to determine the solvent content directly. Unfortunately thermodynamic methods fail because they give only surface excess concentrations and, although the technique of mercury-droplet sampling should work in some instances, it gives gross overestimates in others owing to the tendency of microscopic lenses of solvent to form in the films. These microlenses arise almost certainly because, in equilibrium systems, the final stages of drainage of the film involve a disproportionation that yields the bilayer and a small amount of bulk lipid solution that is temporarily trapped in the film. This bulk solution quickly takes the form of microscopically visible droplets or lenses that drift about in the film for some time before coalescing with the annulus. The lenses do not usually occupy more than a small percentage of the film area [e.g., their presence scarcely affects the capacity per unit area (6, 47, 114)] but they contain a large proportion of the solvent and this is picked up by the mercury droplets. Lens formation is most prominent in systems where highly water-insoluble solvents (e.g., *n*-decane-*n*-hexadecane) are used. In systems containing the more water-soluble and volatile lower homologues (e.g., *n*-hexane-*n*-octane) the aqueous-phase concentration of the alkane is usually sufficiently far below the equilibrium value that the excess solvent in the film dissolves out before lenses can form (6, 47, 114). Although lenses are commonly observed, their presence (or absence) does not affect the solvent contents given in Tables 1 and 2, which are equilibrium values, nor is there any reason to suppose that they significantly affect the water permeability of a black film.

B. Measurement of Water Permeability

When the black-film system is immersed in an ideal aqueous solution of only two components (water and a solute) the fluxes across the membrane are given by irreversible thermodynamics as (78)

$$J_v = L_p(\Delta p - \sigma RT \Delta c_s) \quad (1)$$

$$J_s = c_s L_p (1 - \sigma) \Delta p + [\omega - c_s L_p (1 - \sigma) \sigma] RT \Delta c_s \quad (2)$$

where J_v is the volume flux, J_s is the solute flux, L_p is the filtration coefficient, σ is the reflection coefficient, R is the gas constant, and T is the temperature; c_s is the mean solute concentration, Δc_s and Δp are the solute concentration and hydrostatic pressure differences, respectively, across the membrane, and ω is a permeability coefficient for the solute. Thus a volume flux may arise in two different ways, one because of a finite hydrostatic pressure difference across the membrane and the other through the asymmetric distribution of a solute for which the reflection coefficient is not zero. In practice

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the maximum hydrostatic pressure differences that black films will support produce extremely small volume fluxes of water and in all measurements so far reported, p was effectively zero. Volume fluxes arise therefore almost exclusively from osmotic pressure gradients. Isotopic water fluxes are described by *Equation 2*. Thus if tritiated water is considered to be the solute in normal water, $\sigma = 0$, $\Delta p = 0$ and *Equation 2* reduces to

$$J_s = \omega RT \Delta c_s \quad (3)$$

Two different techniques are required to measure volume and solute water fluxes, respectively; these are described in separate sections below. Neither measurement is entirely straightforward, because in both instances unstirred layers adjacent to the membrane can have resistances comparable to that of the membrane itself.

1. Osmotic fluxes

Apparatus suitable for this type of measurement has been described by a number of authors (25, 42, 47, 56, 67, 74). The bare essentials are to be able to form a membrane that initially separates two aqueous solutions of similar composition and, when the membrane is stable in its area and thickness, to change to a known extent the osmotic pressure on one side only. The resulting volume flow is measured by closing one of the aqueous compartments so that it contains no air and so that changes in its volume can be backed off by means of a micrometer syringe. A suitable apparatus is illustrated in Figure 2, a detailed description of which has been given elsewhere (25, 47). Briefly, black films are formed across one end of a piece of polytetrafluoroethylene (PTFE) tubing. A Hamilton syringe, coupled to a micrometer drive, is attached to the other end of the tubing and creates the required closed compartment of adjustable volume. The steel needle of the syringe and a stainless steel wire in the outer compartment may be used as electrodes to monitor changes in membrane capacity. The whole unit is placed in an air thermostat that can be controlled to about 0.5°C.

The brush or pipette techniques (47, 94, 134) may conveniently be used to form the membranes, which may be inspected and have their areas measured with the aid of a low-power microscope. When the membrane has reached a steady state the external solution is either exchanged or concentrated by the addition of a small volume of solid or highly concentrated impermeable solute. The resulting volume flux of water causes the membrane to bulge inward or outward, as may be seen through the microscope. The micrometer is then adjusted to restore the membrane to its planar state. This operation is carried out at regular intervals and the water flux J_v is determined from a plot of the volume flow against time, examples of which are given in Figure 3, and a knowledge of the membrane area.

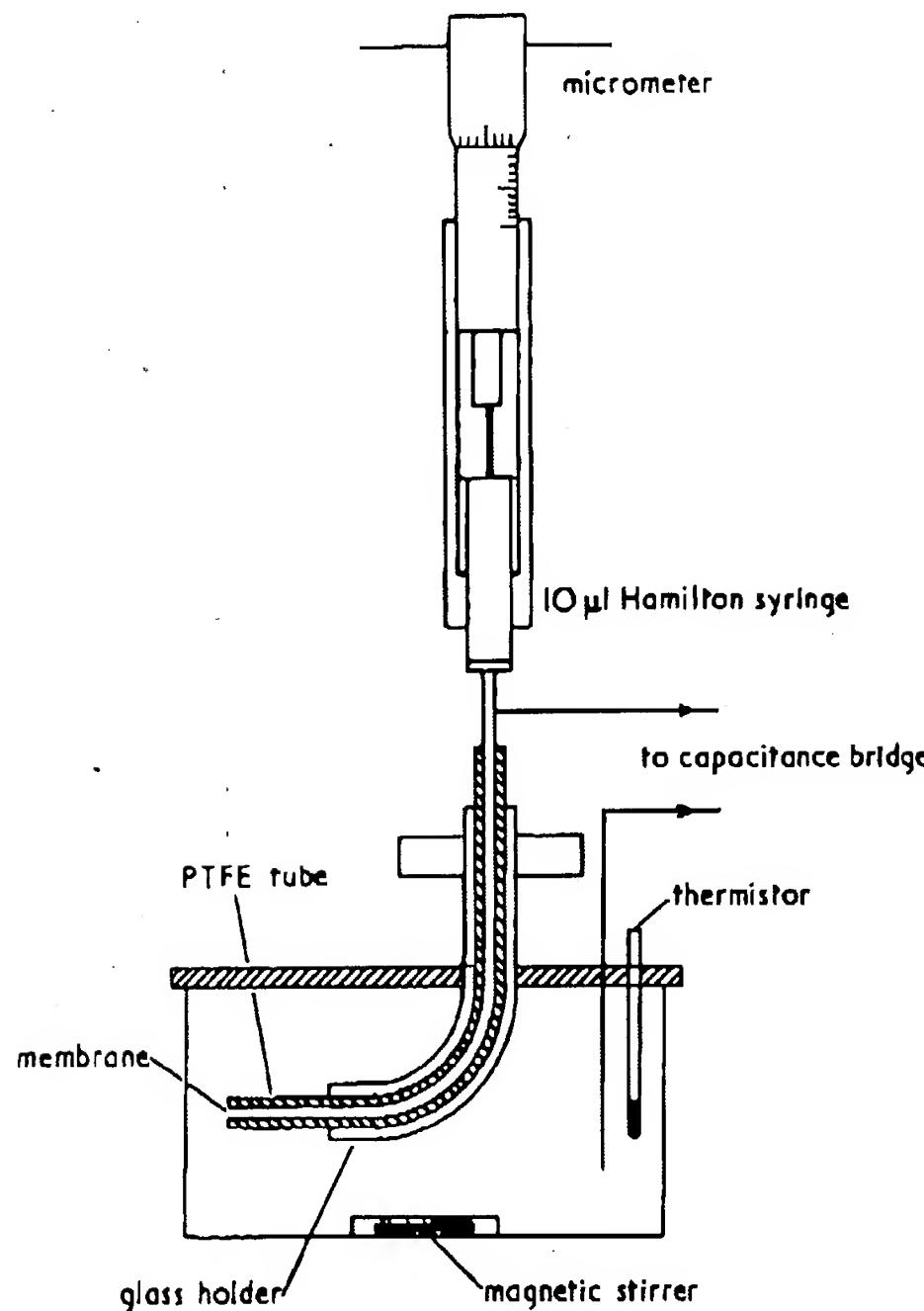


FIG. 2. Apparatus for measuring osmotic water permeabilities of black films.

If the hydrostatic pressure difference across the membrane is taken to be zero and the solute is assumed impermeable (for purely lipid membranes this will be effectively true for solutes such as inorganic salts, urea, glucose, sucrose), i.e., $\Delta p = 0$, $\sigma = 1$, Equation 1 reduces to

$$J_v = -L_v R T \Delta c_s \quad (4)$$

The osmotic water permeability coefficient, normally symbolized as P_o , is de-

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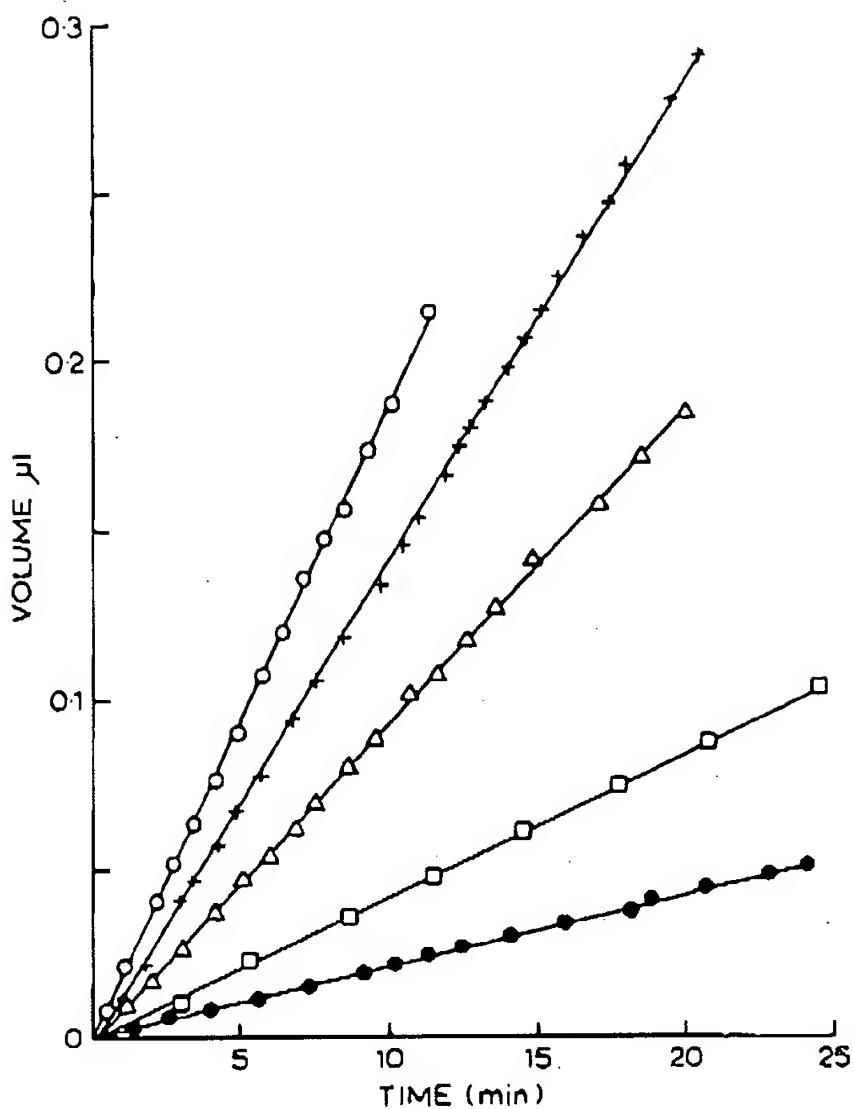
symbolized as P_t , is de-

FIG. 3. Volume flow of water across black films formed from various lipids in decane: \circ , -., egg phosphatidylcholine; Δ dioleyl phosphatidylcholine; \square glyceryl monooleate; \bullet sphingomyelin/cholesterol. Osmotic gradient was 0.3 M NaCl ($T = 25^\circ\text{C}$) for all films except sphingomyelin, where it was 0.47 M NaCl ($T = 30^\circ\text{C}$). Different slopes of plots reflect differing film areas as well as different permeabilities. [From Fettiplace (45).]

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$$P_t = L_v \frac{RT}{V_w} \quad (5)$$

where V_w is the partial molar volume of water. Taking into account nonideality in the aqueous solutions and using Equation 5, Equation 4 may be rewritten

$$J_v = P_t V_w (g_{s2} c_{s2} - g_{s1} c_{s1}) \quad (6)$$

³ P_t is expressed in this form so that it has the units of a permeability coefficient. A P_t of 1 cm s^{-1} is equivalent to a hydraulic conductivity (L_v) of $7.386 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1} \cdot \text{atm}^{-1}$ or to $L_v \cdot RT$ of $18.07 \text{ cm}^4 \cdot \text{s}^{-1} \cdot \text{osmol}^{-1}$, both at 25°C .

where c_{s2} and c_{s1} are the concentrations of solute on the two sides of the membrane and g_{s2} and g_{s1} are the corresponding rational osmotic coefficients. Obviously if $\sigma < 1$ or $\omega > 1$ the interpretation of volume flows is more complicated.

In *Equation 6* c_{s2} and c_{s1} are the solute concentrations at the membrane surfaces and cannot in general be assumed to be equal to the bulk concentrations in the two compartments. This is because the water that passes through the membrane dilutes the solution it enters and concentrates the solution it leaves. Solute concentration gradients thus tend to occur, the size and importance of which depend on their rate of dissipation through backdiffusion of the solute and by the various stirring effects that may be present. If it is assumed that the solute concentration c_s has its bulk value $c_s(b)$ to within a distance δ of the membrane surface, that its value at the membrane surface is $c_s(m)$, and that no stirring occurs in the boundary layer of thickness δ , then for that side of the membrane toward which the water is moving, $c_s(m)$ and $c_s(b)$ are related by the expression (32)

$$c_s(m) = c_s(b) \exp[-J_v \delta / D_s] \quad (7)$$

where D_s is the diffusion coefficient of the solute. The use of *Equation 7* is complicated by the fact that the flux J_v is related to $c_s(m)$ also through *Equation 6*. Using an iterative approach, Hanai and Haydon (56) concluded that in the black-film systems that they studied a neglect of the effects underlying *Equation 7* should give rise to serious errors in the permeability coefficient. [A similar conclusion may be reached from the recent analysis of Pedley and Fischbarg (104)]. However, Hanai and Haydon also pointed out that although the uncorrected permeability coefficients obtained with different solutes were very similar, the application of corrections according to *Equation 7* led to permeability coefficients that depended strongly on the nature and concentration of the osmotically active solute. This finding seemed sufficiently unlikely for it to be suggested that, in the osmotic systems, the effects of unstirred layers may be largely nullified by natural convection (56). Thus the differences between the solute concentrations $c_s(m)$ and $c_s(b)$ would give rise to density gradients that would in turn produce convection and mixing of the solution adjacent to the membrane (56). An approximate theoretical treatment of natural convection during osmotic flow has to some extent confirmed this idea (41). In general therefore errors caused by unstirred layers in osmotic permeability studies on lipid bilayers seem likely to be fairly small. However, whether this is so in any particular instance will depend strongly on the solute used and on the design of the measuring chamber, and of course the errors will become more serious the more permeable the bilayer.

2. Isotopic exchange diffusion

The diffusion of tritiated water across black films has been studied by a number of authors (25, 42, 57, 74, 141-143). The technical problems involved,

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aside from the effects of unstirred layers, are rather less serious than for osmotic flux measurements. The main requirement is simply that the two aqueous compartments should be completely isolated from each other, but that at the same time samples should be obtainable from one or both of them. Other details of the technique have been described elsewhere (47).

Equation 3 may be rewritten

$$J_s = -P_d(c_{s2} - c_{s1}) \quad (8)$$

where $P_d = \omega RT$ and c_{s2} and c_{s1} are the concentrations of the isotope on the two sides of the membrane. Unlike the situation in the osmotic flux measurements, where density gradients appear largely to nullify the effects of unstirred layers, isotope diffusion creates no density gradients and unstirred layers pose a serious problem. The evidence for the presence and thickness of the unstirred layers in black-film systems came originally from a series of experiments in which water fluxes were measured across open holes and holes containing glass meshes (57). Subsequently a great deal more evidence has been accumulated (5, 25, 42, 71, 141-143). As a diffusion barrier is in series with the lipid membrane, the combined unstirred-layer permeability $D_{H_2O}(\delta_1 + \delta_2)$ must be added reciprocally to the membrane permeability P_d . Thus, the apparent permeability of the membrane P , which is obtained from experiment, is given by

$$\frac{1}{P} = \frac{1}{P_d} + \frac{\delta_1 + \delta_2}{D_{H_2O}} \quad (9)$$

From the estimates of $\delta_1 + \delta_2$ it becomes clear that, despite maximal stirring of the bulk phases, the second term on the right-hand side of *Equation 9* is normally comparable to the first. Various methods have been used in attempts to overcome this problem. The first was based on a suggestion by Ginzburg and Katchalsky (53). This approach involves placing in the hole normally occupied by the lipid membrane first one and then two layers of identical membrane—the thinner varieties of cellophane being usually suitable. The permeability to tritiated water is measured in each case, yielding two values, P' and P'' , which may be expressed

$$\frac{1}{P'} = \frac{1}{P_d} + \frac{\delta_1 + \delta_2}{D_{H_2O}} \quad (10)$$

and

$$\frac{1}{P''} = \frac{2}{P_d} + \frac{\delta_1 + \delta_2}{D_{H_2O}} \quad (11)$$

where P_d is here the permeability of a single layer of cellophane. Knowing P' and P'' and D_{H_2O} , *Equations 10* and *11* may be solved for $\delta_1 + \delta_2$. If it is then assumed that the hydrodynamic conditions in the system are independent of whether cellophane or black films are present, this value of $\delta_1 + \delta_2$ may be used to correct

the lipid membrane permeabilities (42, 47). Another method utilizes the supposition that butanol permeates lipid bilayers so readily that flux measurements of this substance, labeled isotopically, yield effectively the permeability of the unstirred layers (71). A third approach, that of Andreoli and Troutman (5), rests on the assumption that the diffusion coefficient of water in the unstirred layers is inversely proportional to the viscosity of the aqueous solutions. When the latter was changed by adding sucrose or dextran a linear relationship was found between $1/P$ (see Eq. 9) and the viscosity and, from the slope of the line, $\delta_1 + \delta_2$ was estimated. The results seem reasonable, but it must be pointed out that physically δ is a very ill-defined quantity and can be given numerical values only through expressions such as Equations 7 and 9. Thus there are no theoretical reasons to suppose that the effective unstirred-layer thickness does not vary with viscosity.

C. Results for Unmodified Bilayers

The results obtained with the two different methods (osmotic and isotopic) for measuring the water permeability are described separately. The osmotic method is dealt with first, and emphasis is placed on the extent to which the membrane constituents can influence the permeability. As assayed by tritiated water transfer the permeability nearly always requires correction for the resistance of the unstirred layers and, without such correction, is less useful for indicating the properties of the membrane itself. As discussed in section II A, the use of black films suffers from the disadvantage that in all but the simplest cases it is difficult to determine the composition of the membrane, particularly when lipid mixtures (e.g., lecithin-cholesterol) are used. Even for membranes formed from a single species of lipid dissolved in a nonpolar solvent, the membrane interior is a mixture of lipid chains and solvent. The relative proportions of lipid and solvent in the membrane differ from that in the film-forming solution and, depending on the type of solvent, there can be up to 50% by volume of solvent retained in the membrane. The presence of this solvent affects the thickness and fluidity of the nonpolar region of the membrane and both properties are likely to influence the water permeability.

1. Osmotic measurements

The commonest type of black film examined is that formed from egg yolk phosphatidylcholine dispersed in an alkane solvent such as *n*-decane. With decane as the solvent, the nonpolar core of the membrane is about 48 Å thick and 30–40% of its volume consists of decane (see Table 2). The osmotic permeability of egg phosphatidylcholine membranes has been measured by different people at a variety of temperatures, and in order that a comparison may be made the values have all been scaled to 25°C by use of measured or assumed activation energies (Table 3). Permeabilities range from about 20 to 50

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TABLE 3. Osmotic permeability coefficients for black films
formed from various lipids

Lipid	Mean Number of Double Bonds per Chain	P_t (Measured), μm/s	Temper- ature, °C	P_t Corrected to 25°C, μm/s	E_a , kcal/mol	Ref.
Egg phosphatidylcholine*	0.81	78.9	36	36		
		86.8	36	40		
		104	36	48		
					(12.7)	74
Egg phosphatidylcholine*		42	36	19	(12.7)	49
Egg phosphatidylcholine*	0.67	18.4	20-22	25	(12.7)	54
Egg phosphatidylcholine	0.73	55.3	37	24	12.96	
Dipalmitoyl (16:0/16:0)						
phosphatidylcholine	0	51.5	37	18‡	18.75	55
Plant phosphatidylcholine	1.73	64.1	37	32	10.76	
Egg phosphatidylcholine	1.2	37.4	25	37.4		
Egg phosphatidylcholine	1.2	36.9	25	36.9	10.8	
Dioleyl (18:1/18:1)						
phosphatidylcholine	1.0	35.8	25	35.3		
Monoolein (18:1)	1.0	51.2	25			
Monoolein (18:1)†	1.0	50	25		14.2	
Monolinolein (18:2)†	2.0	73.2	25			
Egg phosphatidylcholine + cholesterol in film- forming solution						45
Cholesterol 14 mM		30.3	25			
Cholesterol 22 mM		17.2	25			
Cholesterol 28 mM		14.4	25			
Sphingomyelin + 14 mM cholesterol in film- forming solution		3.7	31	2.3	14.9	

All lipids dispersed in *n*-decane except * *n*-tetradecane/chloroform/methanol and † *n*-hexadecane. Egg phosphatidylcholine contains a mixture of chains of different lengths and unsaturation (mean length about 18 carbons, mean unsaturation given in 2nd column). Temperature corrections are based on activation energy E_a . E_a values in parentheses have been assumed, on composition grounds, to be the value of Price and Thompson (107). Mean P_t (egg phosphatidylcholine) = 33.3 μm/s. ‡ Obtained by correcting P_t at 37°C to 25°C with the E_a given.

μm/s with a mean of 33.3 μm/s (Table 3). This corresponds to a variation of 45–112 μm/s at 37°C. Much of this scatter in values may have arisen because of differences between the various lipid samples used. The kinds of differences that could occur include variations in chain composition (egg phosphatidylcholine contains a mixture of alkyl chains of different lengths and unsaturation) and also the presence of contaminating lipids such as sphingomyelin or lysophosphatidylcholine (101, 117). One investigation has avoided this problem by use of a pure synthetic phosphatidylcholine (45).

The fraction of solvent retained in the nonpolar interior of the membrane can be reduced by use of a long-chain alkane such as *n*-hexadecane (Tables 1 and

2). Membranes formed from monoolein dissolved in either decane or hexadecane have similar osmotic permeabilities of about $50 \mu\text{m/s}$ at 25°C (Table 3). The hexadecane membranes retain only about 26% by volume of solvent compared with 49% for the decane membranes (Table 1). The different composition results in the hexadecane membranes being thinner (38 Å compared with 48 Å for decane). It is difficult to interpret the results with the different solvents because the substitution may result in several factors changing simultaneously, each of which could influence the permeability. On the simple idea that water permeation is limited by solution and diffusion of the water in the nonpolar interior of the membrane, changes in thickness and fluidity will both influence the permeability. Thus, although membranes formed with decane as the solvent are thicker, they also contain a considerable amount of lower viscosity solvent in which water might more easily dissolve and diffuse.

If the barrier to the movement of water across lipid bilayers is considered to be the nonpolar interior (this question is examined more closely in sect. IV), variations in the lipid that would alter the fluidity and thickness of the hydrocarbon core might be expected to produce permeability changes. In fact, increasing the degree of unsaturation of the acyl chains of the lipid increases the water permeability (Table 3). Conversely, hydrogenating the lipid or using analogues with saturated chains can reduce the permeability (45, 49, 55). The maximum variation observed by altering the unsaturation is of the order of $\pm 50\%$. Increasing the length of the chains also decreases the permeability (45). Lower permeabilities therefore can be achieved with lipids containing long saturated chains (e.g., 22:0, 24:0, and 24:1) such as those occurring in sphingomyelin (13, 45, 129). Possibly membrane fluidity and water-solubilizing properties can also be influenced by interactions between the lipid head groups and such interactions may therefore affect the water permeability. Graham and Lea (54) have shown that the permeability of phosphatidylserine films is a function of the pH of the aqueous phase (0.1 M NaCl). Increasing the pH from 3.0 to 9.0, which results in a change in the head-group ionization (100), produces an increase in the water permeability.

One common manipulation that alters the water permeability is the addition of cholesterol. This has been demonstrated to produce up to a three- to fivefold reduction in the permeabilities of membranes formed from phosphatidylcholine (45, 49) and other types of lipid (49). The reduction in permeability was graded with the amount of cholesterol added. The major difficulty with this kind of manipulation in black films is that the relative proportions of phosphatidylcholine, cholesterol, and solvent in the membrane are not accurately known under any of the conditions. The addition of increasing amounts of cholesterol produces a graded increase in the capacity per unit area of phosphatidylcholine/decane films from 0.38 to $0.60 \mu\text{F/cm}^2$ (60, 108), and changes in thickness and solvent content appear to be taking place. Attempts made to estimate the amount of cholesterol incorporated in the films have indicated that the cholesterol/lipid ratio in the films is lower than that in the film-forming solution (23, 98).

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measurements on black films usually require good membrane stability, and this can often be achieved by addition of small amounts of cholesterol. Consequently there are a number of estimates of the permeability of phosphatidylcholine/decane membranes containing a little cholesterol. The values obtained are all about 20–30 μ m/s at 25°C (67, 107, 108) and are slightly lower than the mean value of 33.3 μ m/s for the pure phosphatidylcholine/decane membranes. The maximum reduction in the permeability on addition of cholesterol to phosphatidylcholine/decane membranes has been reported to give values of 7.5 μ m/s [36°C (49)] and 14.4 μ m/s [25°C (45)]. The first value is rather low, largely reflecting an abnormally low permeability for phosphatidylcholine in the absence of cholesterol. Tien and Ping Ting (136) found that films formed from oxidized cholesterol or cholesterol films stabilized with a charged surfactant such as dodecyl acid phosphate or cetyltrimethylammonium bromide had permeabilities of 8–9 μ m/s (22.5°C). These permeabilities should be close to that for a pure cholesterol membrane, and the addition of phosphatidylcholine chains would be expected to increase the permeability. Thus 8–9 μ m/s may represent the lower limit to which the permeability of phosphatidylcholine/decane membranes could be reduced by cholesterol addition.

The temperature dependence of the water permeability was originally studied for the light that it might throw on the possible mechanisms for the movement of water across the bilayer (107, 108). When the results were expressed in the form of an Arrhenius plot, a linear relationship was obtained between $\ln P_t$ and the reciprocal of the absolute temperature (107, 108). On the assumption that the permeability coefficient could be expressed in the form

$$P_t = A \exp(-E_a/RT) \quad (12)$$

an activation energy (E_a) could be determined. The values obtained for E_a were 12.7, 13.1 (107), and 14.6 kcal/mol (108) for phosphatidylcholine/decane films containing various amounts of cholesterol. Even though there is some question about the interpretation of these results, since there are changes in film composition and cholesterol content with temperature (108), they are largely in agreement with subsequent experiments on membranes containing no cholesterol (45, 55). In these experiments the activation energy for water transport across phosphatidylcholine/decane membranes lay in the range 10.8–13 kcal/mol, the value depending on the type of phosphatidylcholine used. There was some suggestion from the latter experiments that the more unsaturated the lipid, the lower the activation energy (Table 3; 55).

Since the types of lipid in a membrane can affect its water permeability, it is of interest to ask whether much lower permeabilities can be achieved by an appropriate choice of lipid. Finkelstein and Cass (49) and Holz and Finkelstein (71) have reported permeability coefficients of about 2.5 μ m/s by using a mixture of ox brain lipids plus tocopherol and additional cholesterol. The mixture contained a number of different glycerol- and sphingolipids. Osmotic permeability coefficients of 3–4 μ m/s have been reported for membranes formed from

sphingomyelin and cholesterol (Table 3; 45), and the use of sphingomyelin also produced low tracer permeabilities (48). The high content of long and saturated acyl chains in the sphingomyelin might be partly responsible for the low permeabilities. In addition there could be substantial head-group interaction in sphingomyelin, occurring by hydrogen bonding between the 3-hydroxy groups of the sphingosines (22, 103). This would restrict the chain movement and alter the fluidity of the membrane interior and might lower the water diffusion coefficient compared with phosphatidylcholine. The use of other sphingolipids such as cerebrosides might produce membranes with an even lower permeability.

2. Isotopic measurements

There have been a number of measurements of the diffusion of tritiated water across black-lipid films, and for various lipid systems an apparent permeability coefficient in the range 1–11 $\mu\text{m/s}$ has been obtained (25, 48, 57, 59, 74, 143). Most of these measurements have been accompanied by a determination of the osmotic permeability coefficient P_t for the same lipid under similar experimental conditions and the common finding has been that the coefficient obtained from isotope diffusion is always smaller than P_t , the ratio sometimes being as large as 20 (74). The value obtained for the apparent tracer permeability has also been shown to depend on various experimental conditions such as the geometry of the cell and the degree of stirring of the aqueous phase (25, 42, 57) but in some cases to be independent of lipid variations that have large effects on the osmotic coefficient P_t (74). From these kinds of experiments and others described in section II B, the general conclusion has been that the tracer data do not reflect the true permeability of the membrane, but rather that of the unstirred layers adjacent to the membrane. The tracer permeability is therefore only useful either if an adequate correction can be made for the resistance of the unstirred layers or if the membrane permeability is low compared with that of the unstirred layers. In those situations where an attempt has been made to correct for the unstirred layers, it has been shown that P_t and P_d are equal, to within experimental error (25, 42, 57).

D. Permeability to Nonelectrolytes

The techniques used for measuring the tritiated water flux across black films can also be applied to nonelectrolytes, the permeability being obtained by measuring the flux of labeled solute across the film. The kinds of nonelectrolytes whose permeabilities have been measured in this way include urea, glycerol, erythritol, formamide, and butyramide (86, 105, 143, 148). For all these substances the permeabilities are considerably lower than that for water but, like the water permeability, depend on the lipid composition of the membrane (52, 86). For example, the permeability coefficient for urea in phosphatidyl-

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choline films is about $4 \times 10^{-2} \mu\text{m/s}$ [20–28°C (86, 105, 143)] and in phosphatidylcholine + cholesterol films is about $6 \times 10^{-3} \mu\text{m/s}$ [25°C (48)]. These values are low enough to justify the use of urea as an impermeant solute for osmotic measurements.

E. Pore-Containing Membranes

The ion permeabilities of black films, as indicated by their electrical conductance, can increase enormously on addition of certain polypeptide antibiotics (for reviews see 64, 92). One mechanism by which this increase in permeability is thought to occur is by the formation across the membrane of hydrophilic channels—small holes a few angstrom units in diameter that can accommodate ions and water molecules. For gramicidin A, which is the best example of channel formation, the evidence that pores exist rests largely on the presence of conductance changes that arise from the formation and breakup of the single channels (64, 69). The channels are thought to be about 28 Å long and 4 Å internal diameter (138). Rosenberg and Finkelstein (120) have measured the additional water permeability induced in a black film by the presence of the gramicidin channels and have shown that it is linearly related to the increase in ion permeability as reflected by the electrical conductance of the film. They obtained an additional P_r of $3.42 \mu\text{m/s}$ normalized to a film conductance in 0.01 M NaCl of $10^{-2} \Omega^{-1} \text{cm}^{-2}$. (The background conductance of an unmodified black film is ca. 10^{-8} – $10^{-9} \Omega^{-1} \text{cm}^{-2}$.) Since the conductance per unit channel is known, it is possible to calculate the number of channels per unit area for a given conductance and hence determine the water permeability of a single gramicidin channel. From the measurements mentioned above, this value was determined to be $9.58 \times 10^{-15} \text{ cm}^3/\text{s}$ (120). Thus it is clearly possible to have a membrane with a large ion permeability through aqueous pores without the water permeability being altered much. This reflects the fact that, even when in a highly conducting state, a very small fraction (<0.1%) of the membrane area is occupied by the gramicidin channels.

In contrast to gramicidin, addition of a polyene antibiotic such as nystatin or amphotericin B to black films induces a substantial increase in the permeability to water as well as to ions. Nystatin and amphotericin B have been shown to induce an anion selective increase in conductance (4, 26, 50) and also up to a 20-fold increase in the water permeability of black films containing cholesterol (3, 5, 71), with P_r values as large as about $500 \mu\text{m/s}$ reported for amphotericin B (3). For a membrane conductance in 0.1 M NaCl of $10^{-2} \Omega^{-1} \text{cm}^{-2}$, the normalized osmotic permeability P_r is found to be $40 \mu\text{m/s}$ for nystatin (71) and 18 – $27 \mu\text{m/s}$ for amphotericin B (5, 71). It is generally thought that the polyene antibiotics also produce aqueous channels in the membrane, and recently the measurements of single-channel conductances have been reported for amphotericin B (40). In 0.1 M NaCl, the single-channel conductance is about $2.5 \times 10^{-13} \Omega^{-1}$, which is an order of magnitude smaller than for gramicidin. With this

conductance per channel the water permeability of a single channel can be calculated as $5.6 \times 10^{-14} \text{ cm}^2/\text{s}$. The polyenes have also been demonstrated to increase the permeability of membranes to small polar nonelectrolytes such as urea, ethylene glycol, and glycerol (2, 3, 71). The reflection coefficient increased with increasing molecular diameter, and molecules larger than glucose (radius $\approx 4 \text{ \AA}$) were essentially impermeant (2, 71). The "cutoff" was assumed to be caused by the limit imposed by the dimensions of the channel, which is taken to have an internal radius of $4-5 \text{ \AA}$ (2, 71). The cross-sectional area of the polyene channel is thus about a factor of 5 larger than the gramicidin channel, and this could largely account for the different single-channel water permeabilities for the two types of antibiotic.

Only a small fraction of the membrane area need consist of aqueous channels in order to substantially modify its permeability properties. This point can be illustrated by considering a membrane with a high water permeability, due to amphotericin, of $500 \mu\text{m}^2/\text{s}$. From the permeability per single channel, it can be estimated that this would require about 10^{12} channels/cm², and these would occupy 0.5% of the membrane area.

Isotopic water permeabilities have been measured for pore-containing membranes and, despite attempts to correct for unstirred layers, the measured permeability was found to be lower than that obtained from the osmotic method. For membranes containing polyenes, P_w/P_d was 3.0-3.75 (5, 71); for gramicidin P_w/P_d was 5.3 (120).

III. LIPOSOMES

A. Osmotic-Shock Experiments

At temperatures greater than their phase-transition temperature, phospholipids in water form structures that consist essentially of lipid bilayers completely surrounding aqueous spaces (9, 11, 24). Usually these structures are made up of many layers of bilayer membrane, but their precise shape, size, and number of layers depend strongly on the method of preparation. For example, with minimal mechanical disruption, relatively large droplets (or particles) several microns across and consisting of hundreds of concentric bilayers tend to predominate, whereas it is possible to produce by exposure to ultrasonic radiation a dispersion of very small vesicles some $200-500 \text{ \AA}$ in diameter and with only one or two layers of membrane (73). Regardless of their structural details, the liposomes or vesicles all embody closed shells of lipid bilayer surrounding aqueous compartments and, because the bilayers are poorly permeable to solutes such as inorganic electrolytes, sucrose, and other highly polar molecules and are relatively permeable to water, they tend to shrink or swell when the osmolarity of the external solution is changed. This phenomenon is the basis of one of the methods used to measure the water permeability of the lipid bilayer. Another method, developed more recently, involves the application

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of nuclear magnetic resonance (NMR) spectroscopy to suspensions of the small single-walled vesicles. This method measures exchange diffusional fluxes, as opposed to the net fluxes produced in the swelling or shrinking approach.

A pure phosphatidylcholine liposome that has many lamellae and no significant hole in the middle contains little or no osmotically active solution (10). This is because the leaflet separation is small. If, however, a negatively charged lipid, such as phosphatidic acid, is added to the phosphatidylcholine, an electrostatic repulsion between the leaflets arises, with a range that is inversely proportional to the square root of the electrolyte concentration (10). Thus at low electrolyte concentration the leaflet separation tends to be high and vice versa (10). Liposomes made with charged lipids in low electrolyte concentrations therefore contain relatively large volumes of osmotically active solution between their leaflets and can usually be made to shrink or swell by appropriate changes in the osmolarity of the suspending medium. Figure 4 shows the effect of 4% phosphatidic acid on the volume of phosphatidylcholine liposomes in various concentrations of potassium chloride.

The shrinking or swelling produced by a sudden change in the osmolarity of the external medium may be followed by turbidity measurement. A satisfactory theoretical relationship between the optical extinction and the volume occupied by the phospholipid in a suspension is not available, but the two quantities can be related empirically by using a suitable calibration procedure (10, 111). The permeation of water into or out of the liposomes is fairly rapid and the liposomal suspension and the diluting solution have to be mixed quickly in a spectrophotometer cell. In fact stopped-flow methods are desirable to investigate the full range of permeabilities that can be encountered (16, 110). Whether swelling or shrinking occurs, the internal osmolarity will change rapidly, and obviously, unless the water flux at the instant of mixing can be obtained, inaccurate values of the permeability are likely to result. With a stopped-flow technique, mixing may be accomplished and spectrophotometer readings commenced 100–200 ms after the initial contact of the liposome suspension and the solution (16).

The interpretation of the initial shrinking or swelling rates requires ideally a rather precise knowledge of how the structure and permeability of the liposomes vary as their volume changes. For shrinkage it is generally assumed that both the permeability and effective area remain constant. Reeves and Dowben (109) found from microscopic examination of one- to three-layered vesicles after osmotic shrinkage that extruded filaments or spherules had formed but that these remained attached to the vesicles. Although the manner of the attachment was not described and probably could not be discerned, these observations lend considerable support to the assumption of constant area. Moreover there is at present no evidence that lipid bilayers are appreciably extensible or compressible in directions parallel to the leaflet, so that the assumption of constant permeability is also probably reasonable. For swelling, on the other hand, the situation is less clear. The immersion of liposomes containing radioactive ions or molecules in hypotonic media has been reported to cause a

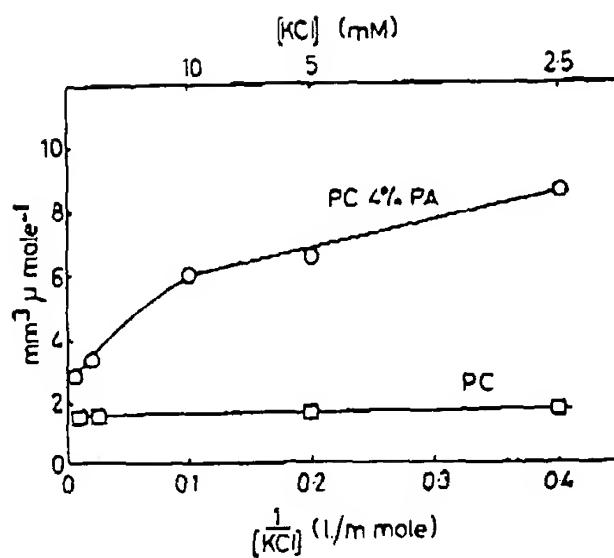


FIG. 4. Innate pellet volumes ($\text{mm}^3/\mu\text{mole}$) of dispersions of phosphatidylcholine (○) and of phosphatidylcholine-phosphatidic acid (mol ratio 96:4) (□) allowed to form in various concentrations of KCl. [From Bangham et al. (10).]

loss of isotope from the structures that is well in excess of the loss that occurs in hypertonic media (10, 110). However, since many of the structures commonly present in a lipid suspension are not necessarily spherical, some swelling in some liposomes might be expected without rupture and release of the contents. Clearly, as soon as a significant proportion of the liposomal material has been ruptured the preparation will not yield reliable permeability data and no doubt for this reason most investigators have concentrated on the shrinkage experiment.

Effectively all the permeability results obtained on systems of the type discussed above are comparative rather than absolute, if only because the surface area of the lipid was not determined. Bangham et al. (10) did determine the surface area of their liposomes, but the interpretation of their data is still complicated by uncertainty about the number of bilayers involved. This latter difficulty may account for the unusually low value of $0.8 \mu\text{m/s}$ at 20°C they obtained for egg phosphatidylcholine containing 4% phosphatidic acid. However, the fact that this result was obtained from a swelling as opposed to a shrinkage experiment may also be relevant. Although the surface area was not reliably known for the liposomes that were shrunk, these experiments yielded the more likely permeability of about $16 \mu\text{m/s}$ (Table 4). Some initial shrinking rates (strictly speaking, initial absorbance changes) of liposomes of different lipid composition have been determined (16, 76) and these values may reflect quite accurately the relative water permeabilities of these systems. It does not seem wholly clear, however, that the initial absorbance changes might not vary somewhat from one lipid preparation to another simply because particle size, shape, or structure varies. In other investigations, activation energies for water permeation have been calculated for different lipid preparations from the temperature dependence of the initial absorbance changes. Again it is not entirely clear whether, at different temperatures, some variation in the rate of the initial absorbance change might not occur even if the permeability of the bilayers remained constant.

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rate pellet volumes (mm^3/ml) of dispersions of phosphatidylcholine (□) and of phosphatidylcholinolipid (○) and of phosphatidylcholinolipid:cholesterol (△) and of phosphatidylcholinolipid:cholesterol:cholesterol (■) were measured to form in various dispersions of KCl. (From Bangham et al., 1964)

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TABLE 4. Water permeabilities for liposomal preparations

Lipid	Form	Method	Permeability, $\mu\text{m/s}$	Temperature, $^{\circ}\text{C}$	Permeability Corrected to 25°C, $\mu\text{m/s}$	E° , kcal/mol	Ref.
Egg phosphatidylcholine + 4% phosphatidic acid	Large multilayered liposomes	Osmotic swelling and shrinking	0.8-16	20	1.0-20	8.25*	10
Egg phosphatidylcholine	Large vesicles (1-2 bilayers)	Osmotic shrinking	40.6-48.9	25	40.6-48.9	8.25 ± 0.25	110
Dipalmitoyl phosphatidylcholine	Small (ca. 400 Å) single-walled vesicles	Exchange diffusion (^1H NMR)	68.5-90.6 16-18 1.7	37 44 20	6.8-7.7 2.6	8.5* 15 ± 1	1
Egg phosphatidylcholine: cholesterol (molar ratio 1:1)	Small (ca. 500 Å) single-walled vesicles	Exchange diffusion (^{18}O NMR)	8	22	9.8	12 ± 2	61
Egg phosphatidylcholine + 4% phosphatidic acid + 50% (mol/mol) cholesterol	Large multilayered liposomes	Osmotic shrinking	8-25 ($>T_c$)	8-25	10.6 ± 0.4 15.0 ± 0.5	10.6 ± 0.4	
Dioleoyl phosphatidylcholine + 4% phosphatidic acid + 50% (mol/mol) cholesterol	Large multilayered liposomes	Osmotic shrinking	$>T_c$	$>T_c$	11.4 ± 0.5	11.4 ± 0.5	
Dipalmitoyl phosphatidylcholine + 4% phosphatidic acid + 50% (mol/mol) cholesterol	Large multilayered liposomes	Osmotic shrinking	$>T_c$	$>T_c$	12.7 ± 0.4	12.7 ± 0.4	
Dipalmitoyl phosphatidylcholine + 4% phosphatidic acid + 50% (mol/mol) cholesterol	Large multilayered liposomes	Osmotic shrinking	43.8 ($>T_c$) 44.0	43.8 ($>T_c$) 44.0	12.5 ± 0.7 10.2 ± 2.7 21.4 ± 4.3 20.9 ± 0.8	12.5 ± 0.7 10.2 ± 2.7 21.4 ± 4.3 20.9 ± 0.8	18
Dipalmitoyl phosphatidylcholine + 4% phosphatidic acid + 50% (mol/mol) cholesterol	Large multilayered liposomes	Osmotic shrinking	34.1 ($<T_c$) 30.7	34.1 ($<T_c$) 30.7	28.3 ± 1.0 21.9 ± 0.4	28.3 ± 1.0 21.9 ± 0.4	

* Assumed value.

The most thorough examination of the absolute water permeability of lipid vesicles, by the osmotic technique, has been done by Reeves and Dowben (110). An important difference between this study and those discussed above was the nature of the lipid preparation, which consisted of roughly spherical vesicles of approximately $1 \mu\text{m}$ diameter and wall thicknesses of only a few (ca. 1-3) bilayers. The preparation and characterization of these vesicles are described in an earlier paper (109). No acidic or otherwise net charge lipid was added to the egg phosphatidylcholine used and the large aqueous volume inside the vesicles was assumed to consist of the 0.2 M sucrose solution in which the dispersion was prepared. A theoretical expression for the turbidity of homogeneous spheres (79, 139) was applied to the vesicle suspension and was found to account tolerably well for variations in the turbidity with vesicle concentration, refractive index change, and wavelength of the light used. The vesicle suspensions were mixed in a stopped-flow apparatus with hypertonic solutions and the resulting shrinkage was followed in the usual way by measuring optical extinction. Shrinkage was assumed to proceed until the osmolarities of the inner and outer solutions were equal. The time courses of the observed extinction changes were compared with curves predicted from theory (see Fig. 5) and from calibration experiments, and

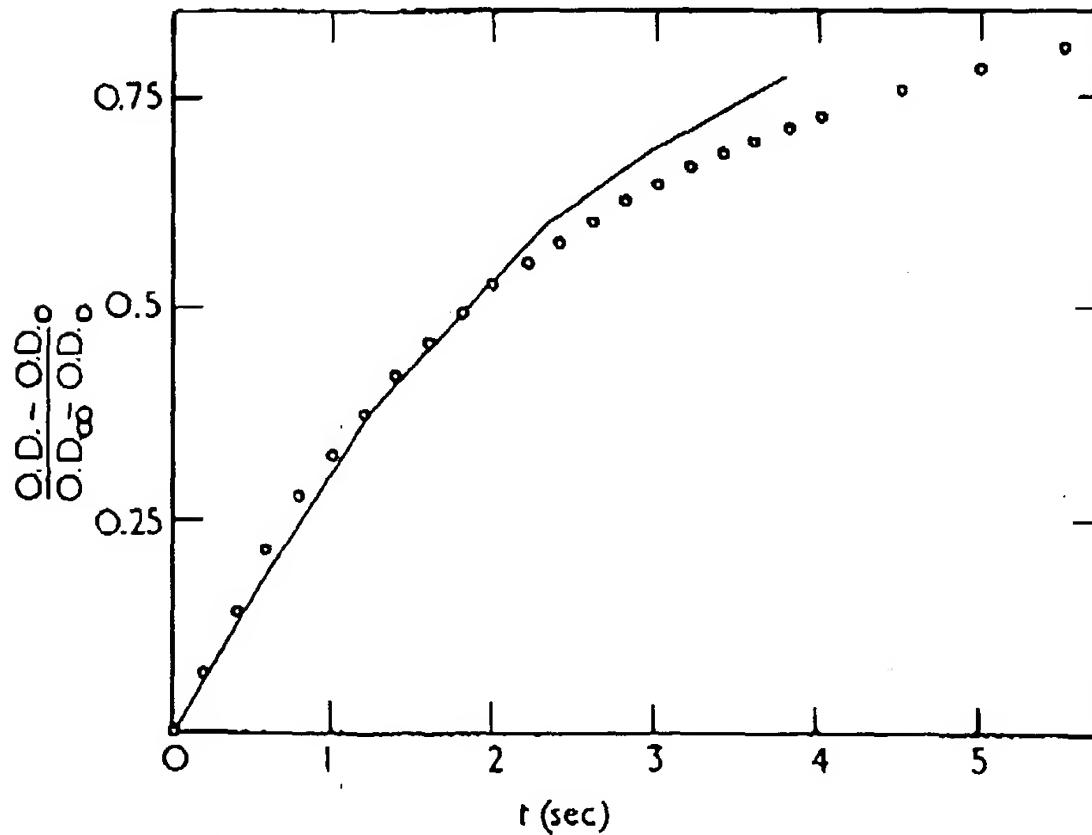


FIG. 5. Experimental and theoretical optical-density changes during shrinkage of egg phosphatidylcholine vesicles. Permeability coefficient of $68.5 \mu\text{m/s}$ ($T = 37^\circ\text{C}$) was chosen so that theoretical curve (solid line) and experimental curve (circles) would coincide at half time. [From Reeves and Dowben (110).]

water permeability of lipid vesicles and Dowben (110). The vesicles discussed above was the roughly spherical vesicles of only a few (ca. 1-3) vesicles are described in the lipid was added to the volume inside the vesicles which the dispersion was homogeneous spheres (79, and to account tolerably concentration, refractive index suspensions were mixed in and the resulting shrinkage extinction. Shrinkage was and outer solutions were changes were compared with vibration experiments, and

the permeability coefficients were calculated. The results are shown in Table 4. The uncertainties in the data are discussed by the authors. They point out that light scattered at low angles from the vesicles may have reached the photomultiplier and that, if so, the permeability coefficient could have been overestimated. On the other hand, the permeabilities shown were calculated assuming single-walled vesicles, whereas there was evidence that some vesicles had more than one layer. This error could lead to an underestimate of the permeability. Stagnant water layers were also considered but, as pointed out in section II B, it is unlikely that errors arising in this way would be significant. It is concluded that the major sources of error act in opposite directions but to extents that are not easy to estimate.

B. Spectroscopic Techniques

The use of NMR spectroscopy to determine the water permeability of lipid vesicles rests principally on the fact that lipid bilayers are very impermeable to inorganic ions. Thus if the paramagnetic ion Mn^{2+} , which greatly reduces the relaxation times of 1H and ^{17}O , is placed either inside or outside the vesicles, it enables the water molecules in the two regions to be distinguished from each other. Since, however, the water may exchange relatively freely across the vesicle membrane, the observed relaxation times are a function of the mean residence time of the water molecules in the vesicles and this in turn is a function of the vesicle radius and permeability. Andrasko and Forsén (1) examined the 1H relaxations in systems in which 43 mM or more Mn^{2+} was trapped inside the vesicles. As the authors point out, such a high concentration of divalent ions is not wholly desirable, since it may affect the bilayer water permeability. Haran and Shporer (61) made use of the greater sensitivity of ^{17}O to Mn^{2+} and were able to work with 5 mM solutions. Thus, for a vesicle suspension in which the continuous aqueous phase contains $MnCl_2$ and therefore yields the short relaxation times, Haran and Shporer give the mean lifetime (τ) of the water molecules within the vesicles as

$$\frac{1}{\tau} = \frac{(1/T_{20} - 1/T_2)(1/T_2 - 1/T_{21})}{(P_0/T_{20} + P_1/T_{21} - 1/T_2)} P_0 \quad (13)$$

where $1/T_2$ is the modified relaxation rate of the water within the vesicle (i.e., the longest component of the ^{17}O transverse relaxation rate) as a result of the exchange with the external solution, $1/T_{20}$ is the relaxation rate of water in the presence of the Mn^{2+} , $1/T_{21}$ is the relaxation rate of pure water, P_1 is the fraction of water within the vesicles, and P_0 is the fraction of water in the external solution. In the suspensions examined, $P_1 \ll P_0$ and $1/T_{21} \ll 1/T_{20}$ so that

$$1/\tau \approx 1/T_2 - 1/T_{21} \quad (14)$$

ges during shrinkage of egg ($T = 37^\circ C$) was chosen so that old coincide at half time. (From

If the volume V and surface area A of the vesicles is known, then the water permeability coefficient P_d is given by

$$P_d = (V/A)(1/\tau) \quad (15)$$

and if the vesicles are spherical, by

$$P_d = r/3\tau \quad (16)$$

where r is the vesicle radius. Note that it is the equilibrium or exchange-diffusion coefficient that is obtained from this technique. Vesicle characterization in this investigation was by electron microscopy and by the addition of PrCl_3 , which produces an upfield shift in the NMR signal of the choline group of the phospholipids. This allowed estimation of the proportions of external and internal surface in the preparation, thus providing further evidence as to how many of the vesicles were single walled. Permeabilities and activation energies determined by Andrasko and Forsen and by Haran and Shporer are given in Table 4.

C. Results

The few absolute values for bilayer water permeability determined for liposomal or vesicular preparations are shown in Table 4. The permeabilities have been given both at the temperature of the experiment and for 25°C by the use of an activation energy from another source, if necessary. Comparisons within this set of data are not easy because, with one exception, different lipids have been used in each instance. In addition the first value should probably be discounted since the number of lipid layers involved is not known and could be quite large. The second and fifth values are remarkably similar to several of the black-film results (Table 3) and suggest that the presence of cholesterol substantially reduces the water permeability, a conclusion further confirmed by comparative studies described below. The third and fourth values are for dipalmitoyl phosphatidylcholine above and below its phase-transition temperature ($T_c = 41^\circ\text{C}$). Even after normalizing the results to 25°C, the permeability for the (hypothetically) fluid leaflet is significantly larger than that for the leaflet in the gel state, as might have been expected, although, as will be seen, other approaches suggest that this difference should be greater. Also the absolute value of the permeability for dipalmitoyl phosphatidylcholine for $T > T_c$ is lower in relation to the egg phosphatidylcholine value than is indicated by other (comparative) data, and both inconsistencies might be a consequence of the fact that appreciable concentrations of Mn^{2+} were present in the vesicles.

Determinations of activation energy and nonabsolute permeability measurements (such as initial shrinking rates) are more plentiful. From the comparison of initial shrinking rates for a wide range of systems Bittman and

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Blau (16) reached a number of interesting conclusions. For dipalmitoyl phosphatidylcholine the water permeability appears to increase by more than 20-fold between 38°C and 45°C, i.e., as it passes through its phase-transition temperature. If the activation energy for this system is taken to be that determined by Blok et al. (17), the 45°C value can be converted to the value that the liquid crystalline leaflet would have at 38°C were it not to revert to the gel phase. This suggests that the phase transition alone increases the permeability by about 17-fold at this temperature. Again, the calculated hypothetical permeability of the liquid crystalline leaflet at 25°C is only about one-third that of egg phosphatidylcholine at this temperature. It is not surprising therefore that Bittman and Blau find that on adding dipalmitoyl phosphatidylcholine to dioleoyl phosphatidylcholine at 25°C there is a marked reduction in the water permeability, which is attributed to the decrease in the concentration of double bonds in the membrane—a conclusion that has also been reached from black-film experiments (sect. II).

The large changes in water permeability that occur at the gel-liquid crystalline phase transition of a lipid have received considerable attention from Blok et al. (17). The increase in the initial shrinkage rate for dipalmitoyl phosphatidylcholine between about 38°C and 42°C was similar to that reported by Bittman and Blau (16), but Blok et al. (17) also show that there is a large discontinuity in the activation energy in the region of the transition temperature. Above T_c , activation energies of 7–11 kcal/mol were found, as opposed to values of 20–28 kcal/mol below T_c (Table 4). This latter value is considerably larger than that calculated by Andrasko and Forssen (1) from their exchange-diffusion data for ostensibly the same system. Since for egg phosphatidylcholine + cholesterol (a system essentially above any phase transition) the activation energies for exchange diffusion and osmotic shrinkage are very similar (Table 4), the question arises as to whether for lipids in the gel state the two methods measure the same quantity.

The addition of cholesterol to egg phosphatidylcholine liposomes was shown by Bittman and Blau (16) and by Jain et al. (76) to produce a roughly threefold reduction in the initial shrinkage rate at 1:1 mol ratios. This is qualitatively similar to the results of black-film experiments (sect. II). Modifying cholesterol at the 3 position to give 3-hydroxycholestene and 3-thiocholestene reduced the potency of small proportions of the steroid, but at the 1:1 mol ratio the differences from cholesterol were small. The influence of cholesterol on the activation energy for water permeation in a variety of lipid preparations and especially the relative effects it has above and below the phospholipid phase transitions have been studied by Blok et al. (18). When added in about 30% (mol/mol), or more, to either dimyristoyl or dipalmitoyl phosphatidylcholine, cholesterol abolished the abrupt changes in water permeability and activation energy for permeation, which normally occur at the transition temperature. Above the phase-transition temperature, cholesterol produced a substantial increase in the activation energies for the above-mentioned lipids (Table 4). This effect was in contrast to the change in the activation energy on addition of cholesterol to egg

or dioleoyl phosphatidylcholine, where the increases were much smaller (Table 4). Below the phase-transition temperature, the addition of cholesterol to dipalmitoyl phosphatidylcholine had very little effect on the activation energy.

IV. DISCUSSION

A. Comparison Between Black-Film and Liposome Results

The chief advantage of the use of black-lipid films for water permeability measurements is that they have accurately known areas and hence precisely calculable permeabilities. The chief disadvantages are that their lipid composition is restricted by the requirement that they form stable membranes; that the amounts of the various lipids present in multicomponent membranes is not easy to estimate; and that of necessity they contain some, though possibly small, amounts of solvent. All these disadvantages may be overcome, at least in principle, by the use of liposomes or vesicles. These, however, have the disadvantages that in osmotic experiments there are complex geometrical changes that are not properly understood, that volume changes have to be studied by essentially empirical optical methods, and that uncertainty in the number of layers of leaflet involved precludes the calculation of accurate permeabilities. Spectroscopic experiments on small single-layered vesicles appear to suffer only from the uncertainty in the area of vesicle surface under examination, but then these methods measure only the equilibrium or exchange-diffusion coefficient P_{e} , not the coefficient of net flux P_i .

Despite the lack of an ideal experimental system, there is considerable agreement among the results obtained from the different preparations and different techniques. As shown in Table 3, the water permeabilities of monoolein membranes formed with decane and with hexadecane are very close. This might originate from the possibly lower viscosity of the decane film being offset by the greater thickness. For present purposes, it is assumed that this argument can be extended to the case of zero solvent content, thus allowing a direct comparison between the black-film and liposome results. This procedure should be regarded warily, however, because the exclusion of the last vestiges of solvent may have a disproportionate effect on the permeability, as discussed later.

With these reservations in mind, the mean permeability of $33.3 \mu\text{m/s}$ ($T = 25^\circ\text{C}$) for egg phosphatidylcholine black films is in reasonable agreement with the values of 40.6 – $48.9 \mu\text{m/s}$ obtained by Reeves and Dowben (110) for egg phosphatidylcholine liposomes. The activation energy for water transport is slightly lower for egg phosphatidylcholine liposomes [8.25 kcal/mol (110), 10.6 kcal/mol (18)] than for black films [10.8 kcal/mol (45), 12.96 kcal/mol (55)]. A reduction of the permeability of egg phosphatidylcholine by incorporation of cholesterol has been observed in both types of bilayer preparation, each giving a limiting permeability at high cholesterol content of the order of $10 \mu\text{m/s}$ at 25°C (45, 61). The two systems may not be strictly comparable, however, since

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were much smaller (Table 1) than that of cholesterol to that of the activation energy.

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for water permeability are and hence precisely these are that their lipid form stable membranes; component membranes is in some, though possibly be overcome, at least in these, however, have the more complex geometrical changes have to be that uncertainty in the calculation of accurate single-layered vesicles of vesicle surface under only the equilibrium or of net flux P .

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permeability of $33.3 \mu\text{m/s}$ in reasonable agreement and Dowben (110) for egg yolk for water transport is [8.25 kcal/mol (110), 10.6], 12.96 kcal/mol (55)]. A oline by incorporation of preparation, each giving a order of $10 \mu\text{m/s}$ at 25°C parable, however, since

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interactions between the phospholipid and cholesterol molecules that occur in the liposomes could be affected by solvent in the black films.

There is also qualitative agreement between the results for the two systems on the effects of varying the unsaturation of the alkyl chains. On the other hand, there are quantitative differences for the permeabilities of saturated lipids. Thus measurements on liposomes formed from dipalmitoyl phosphatidylcholine suggest that there is a big drop in permeability on taking the temperature below the phase transition, which is at 41°C (1), whereas the permeability measured for black films formed from dipalmitoyl phosphatidylcholine at 37°C is not particularly low (55). This discrepancy may be due to the solvent in the black film, which may lower the transition temperature by more than 4°C (see, e.g., 77). Alternatively, there could be patches of the black film containing phospholipid in the gel state and patches of liquid chains and solvent. Thus, besides altering the transition temperature of the lipid, the hydrocarbon solvent could also be instrumental in producing a permeability in parallel with that of the alkyl chains of the lipid. If the lipid itself had a very low permeability, the permeability of the black film could then be greatly affected by the solvent. These kinds of argument also apply to measurements on other impermeable bilayers. Thus, for example, the permeability of the bilayers formed from sphingomyelin may be overestimated on the basis of the black-film results, since the transition temperature for bovine brain sphingomyelin is in the region of 20 – 45°C (13, 129). Nevertheless, apart from these reservations over the measurements on impermeable bilayers, there is reasonable agreement between the liposome and black-film results.

B. Mechanisms of Water Transport

As yet there is no conclusive evidence for the mechanism by which water crosses lipid or lipid + hydrocarbon bilayers. The following discussion is therefore necessarily speculative and inconclusive.

At the outset it is convenient to divide the existing possibilities into two types: those that assume water is aggregated in the bilayer such that it forms more or less continuous files stretching from one aqueous phase to the other (74), and those that assume the water is completely dispersed, as in dilute solution (56).

It is assumed that files might occur at dislocations or discontinuities in the lipid and that the permeabilities of these regions would be very much higher than other regions of the bilayer. There is no direct evidence for the existence of such files and the following, admittedly rather weak, arguments are not in their favor. If the total amount of water per unit area of the membrane is assumed to be similar to that in bulk hydrocarbon of similar chain length to that of the lipid molecules; and all of this water were in the form of files one molecule in width, then there would be about 10^{10} files or pores/ cm^2 . Furthermore, if the water molecules in these pores were able to rotate and exchange protons with their

neighbors as they do in bulk, it seems likely that the electrical resistance of the bilayers would be several orders of magnitude less than the observed value—which corresponds roughly with that expected for wet bulk hydrocarbon. Another argument against the existence of pores is the high activation energy observed for water transport. If the diffusion of water in the pores were as in bulk solution, the activation energy would be 4.6 kcal/mol (144) rather than the values of 8–15 kcal/mol given in Tables 3 and 4. Finally it is pertinent that the permeability for diffusional exchange of water (P_d) seems to be similar, if not equal, to the permeability for net movement (P_n). This implies that there is no significant interaction between water molecules crossing the membrane (65, 66) and hence that there is in general no more than one molecule at a time in the pores. This clearly does not support the type of filing mechanism discussed above.

In the second type of mechanism the membrane is assumed to be a homogeneous layer in which water dissolves and moves across by diffusion. There are two possible rate-limiting steps in this process: 1) the transport in the interfacial region between the membrane and the aqueous solution and 2) diffusion in the nonpolar interior of the membrane. If the diffusion and concentration of the water within the bilayer are uniform, and the diffusion is isotropic, P may be described by the following equation (149)

$$1/P = (h/KD) + (2\lambda/D_{sm}) \quad (17)$$

where K is the partition coefficient, D is the diffusion coefficient of water within the membrane interior, h is the thickness of the interior, λ is the width of each interfacial region containing the polar head groups of the lipids, and D_{sm} is the diffusion coefficient across this interfacial region from the aqueous solution to the membrane. The first and second terms on the right-hand side of Equation 17 are the diffusional resistances of the membrane interior and the interfacial regions, respectively. Strictly, to account for the inhomogeneity of the interior of the membrane, the first term should be replaced by the integral (33)

$$\int_0^h \frac{dx}{K(x)D(x)}$$

where x is distance across the bilayer.

There is no simple way to decide which of the two resistance terms is the most important in limiting the permeability. The observation that the water permeability of black films can be altered by varying the chain composition but keeping the head group the same (45, 55) is consistent with the idea that the interior constitutes the main barrier to movement. This argument is strengthened for the monoglycerides, where it is known that the area per glycerol head group does not change detectably as the chain length or unsaturation is altered (45) and, at least for the change in chain length, there are no major changes in solvent content or composition of the films (45).

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electrical resistance of the bilayer is less than the observed resistance expected for wet bulk. The resistance of pores is the high resistance of water in the bilayer, which would be 4.6 kcal/mol (144). For pores of radii 3 and 4. Finally it is assumed that the permeability of water (P_w) seems to be limited by the rate of movement (P_i). This implies that the water molecules crossing the bilayer do so more than one molecule at a time, suggesting a type of filing mechanism.

It is assumed that the water molecules cross the bilayer by diffusion. This is done partly because: 1) the transport in the bilayer is faster than in the aqueous solution and 2) the diffusion coefficient of water is higher than that of the hydrocarbon. If the diffusion and partition coefficients are the same, and the diffusion is limited by the rate of movement, then the diffusion is limited by the rate of movement (149).

(17)

coefficient of water within the bilayer, λ is the width of each layer of the bilayer, and D_{sm} is the diffusion coefficient of water in the aqueous solution to the bilayer. The left hand side of Equation 17 is the sum of the resistance of the nonpolar interior and the interfacial region. The heterogeneity of the interior is taken into account by the integral (33).

The argument that the water resistance terms is the same as the resistance of the water in the chain composition but different from the resistance of the hydrocarbon. This argument is based on the assumption that the area per hydrocarbon molecule is the same as the chain length or the same as the chain length, there are more hydrocarbon molecules in the films (45).

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Dix et al. (36) have recently concluded that the movement of certain small nonelectrolytes across the bilayer is dominated by the interfacial resistance. They used electron spin resonance (ESR) spectroscopy to measure the mobilities and partition coefficients of various nitroxide solutes in phosphatidylcholine liposomes. The nitroxide molecules are larger than water, however, and are to some extent amphiphilic. Both factors could result in the nitroxide solutes spending a relatively long time in the interface. It is not obvious that the same conclusions would be reached for water molecules.

Although there is no definitive evidence available at present that clearly establishes where the rate-limiting step in water transport is located, it is assumed for the purpose of the analysis given in the next section that the nonpolar interior is limiting. This is done partly because the circumstantial evidence favors this mechanism and partly because it is at least possible to estimate theoretically what the permeability might be, based on a knowledge of diffusion and partition coefficients of water in liquid hydrocarbons. The properties of the interfacial region are more difficult to model. Conceivably the resistances of both regions make a contribution toward the permeability. If this were the case, however, it might be expected that the Arrhenius plot of $\ln P$ against $1/T$ would not be linear; i.e., there would not be a single activation energy for water permeation (107).

C. Predictions Based on Solubility and Diffusion Mechanism

If it is assumed that the water crosses the bilayer by solution and diffusion in the nonpolar interior, and it is also assumed that the rate at which water traverses the interfacial region is fast compared with this process, only the first term in Equation 17 need be considered, and this can be written as follows (56).

$$P = (D/h)(X_w)(\bar{V}_w/\bar{V}_n) \quad (18)$$

where D and X_w are the diffusion coefficient and mole fraction of water in the nonpolar interior, of thickness h . \bar{V}_w is the partial molar volume of water and \bar{V}_n is the partial molar volume of hydrocarbon in the membrane. No values are yet available for the diffusion and partition coefficients of water in the membrane, but they are available for long-chain saturated hydrocarbons up to hexadecane. For the purpose of this analysis therefore, it is also assumed that the membrane interior can be treated like a layer of bulk liquid hydrocarbon, and the bulk values are used for D , X_w , and \bar{V}_n . The theoretical permeability properties of a bulk layer of liquid hexadecane 30 Å in thickness are given in Table 5. These have been calculated on the basis of Equation 18, with the two sets of values for the different water solubilities as determined by Schatzberg (123, 124) and Englin et al. (39). For comparison the measured water permeabilities are given for phosphatidylcholine liposomes and black films formed from dipalmitoyl phosphatidylcholine (above its transition temperature) and also for monolein/hexadecane black films. For all

TABLE 5. Comparison of theoretical and experimental permeabilities

System	Permeability, $\mu\text{m}^2/\text{s}$		E_a , kcal/mol	Ref.
	25°C	45°C		
Layer of liquid hexadecane 30 Å thick*	99 58	404 190	13.3 11.3	39 123, 124
Dipalmitoyl phosphatidyl- choline liposomes		18		1
Monolein/hexadecane black films	50		14.2	45
Dipalmitoyl phosphatidyl- choline/decane black films		55†	13.75	55

Theoretical permeabilities were calculated from Equation 18. D was taken as diffusion coefficient of water in liquid *n*-hexadecane [$4.16 \times 10^{-5} \text{ cm}^2/\text{s}$ at 25°C and $5.97 \times 10^{-5} \text{ cm}^2/\text{s}$ at 45°C (124)]; V_h was taken as molar volume of hexadecane (294 cm^3). * The 2 sets of values refer to differences in measured partition coefficients between water and *n*-hexadecane. † Value extrapolated with the given E_a and the measured permeability at 87°C.

the examples, the theoretical permeabilities are higher than the measured ones, and this seems to be consistent with the hydrocarbon chains in the lipid bilayer being more restricted in their motion than in liquid hexadecane, thus lowering the diffusion coefficient of water in the membrane. Although the theoretical and measured permeabilities agree best for monolein, the discrepancy here might be larger if the diffusion and solubility values for the corresponding unsaturated hydrocarbon, *cis*-9-heptadecene, were available. Finkelstein and Cass (49) found that the water solubility in 1-hexadecene was about 15% higher than in the saturated hydrocarbon.

The water permeability of a lipid bilayer has also been calculated for a solution and diffusion mechanism by Träuble (137). He proposed that water crossed by transient holes or pockets of free volume that were due to the thermal motion of the hydrocarbon chains. The diffusion coefficient of the holes was calculated to be about $10^{-5} \text{ cm}^2/\text{s}$, and thus the theoretical water permeability was smaller than those in Table 5. Although it seems probable that water is accommodated within the bilayer through the presence of kinks in the chains, Träuble's picture of water "hitchhiking" across the membrane in the kinks seems less likely to be correct. Thus, it is not known to what extent kinks actually cross the membrane nor is it known whether water molecules may hop from one kink to another. Certainly the interior of a bilayer above its phase transition is now known to be much more disordered than in the illustration in Träuble's paper.

A number of investigators (107, 108, 110) pointed out that the observed value for the activation energy for water permeation (E_a) is consistent with the mechanism in which water crosses by dissolving and diffusing in the nonpolar core. On this mechanism E_a should be equal to the sum of the activation energy for water diffusion and the enthalpy of the partition coefficient of water between

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meabilities

E_a kcal/mol	Ref.
13.3	39
11.3	123, 124
	1
14.2	45
13.75	55

as taken as diffusion coefficient $5.97 \times 10^{-3} \text{ cm}^2/\text{s}$ at
* The 2 sets of values
hexadecane. † Value

the aqueous phase and the membrane interior. Values of these parameters are available for liquid *n*-hexadecane, and from them E_a can be calculated to be 11.3 kcal/mol with the results of Schatzberg (123, 124) and 13.3 kcal/mol with the results of Englin et al. (39). These are in reasonable agreement with the experimentally determined activation energies (see Tables 3 and 5). The enthalpy of solution is reduced by hydrocarbon unsaturation, and this would be consistent with the correlation of E_a and lipid unsaturation (see Table 3).

On the basis of the solubility and diffusion model, the water permeability might be expected to be susceptible to the length and unsaturation of the lipid chains. Englin et al. (39) showed that the solubility of water in bulk hydrocarbon was increased by unsaturation, but was only weakly dependent on the length of the hydrocarbon molecule. No comparable data are available for the diffusion coefficient. Empirical approaches to water diffusion in organic solvents have represented the diffusion coefficient as being inversely proportional to the viscosity of the solvent (56), which for long-chain hydrocarbons increases markedly with chain length at room temperature (121).

D. Environment of Lipid Chains and "Bulk Hydrocarbon" Assumption

Both for the above theoretical analysis of the water permeability of lipid bilayers and for the interpretation of capacity data and the compositions of black films, it has been assumed that the nonpolar core of the membrane has the properties of bulk lipid hydrocarbon. The properties of particular interest are the density, the water solubility (both of which are reflected to some extent in the dielectric constant), and the fluidity.

No precise measurements appear to have been made on the partial molar volumes of methylene groups in lipid bilayers. Comparable information is, however, available for surfactant micelles (30). Here it was found that the mean volume per CH_2 was about 3% larger than in bulk liquid hydrocarbons. The micelles in question were globular and the constraints on chain packing were therefore probably more severe than in planar bilayers. Therefore the hydrocarbon density in phospholipid bilayers probably is very close to bulk values.

The amount of water in a bilayer, and its distribution, is obviously important for the understanding of the water permeability. Suggestions have occasionally been made that more water may be present than that expected from its solubility in bulk hydrocarbons. The evidence for this is not very strong, however. For instance it could be argued, on the basis of the results for surfactant micelles mentioned earlier, that the partial molar volume of the methylene groups in the bilayer is about 3% larger than in bulk hydrocarbons and that the additional 3% free volume is occupied by water. On the other hand, liquid hydrocarbons always have at least 20% free volume (20), only a minute fraction of which can be occupied by water, and thus a further 3% would be very unlikely to make an appreciable difference to the water solubility. It has been argued that water penetrates the outer regions of the hydrocarbon layer. One

such argument arose from a finding that in Montal-Mueller "solventless" bilayers, the electrical capacity was $0.9 \mu\text{F cm}^{-2}$ (93). For this capacity to give the same leaflet thickness as that found from X-ray diffraction it is necessary to assume a dielectric constant of 2.65 rather than that for *n*-alkanes, i.e., about 2.1. The high value for the dielectric constant was taken to indicate that water penetrated into the chains of the leaflet. It has now been shown, however, that this result is incorrect (15, 45) and that the true value is about $0.74 \mu\text{F cm}^{-2}$, which with a dielectric constant of 2.2, is entirely consistent with the X-ray data (Table 2).

Spectroscopic evidence for surfactant micelles (27, 28, 95, 96) shows that all the segments of the surfactant chains are to some extent exposed to water, but it is not clear that water penetrates the micelles. Thus a considerable amount of hydrocarbon is necessarily at the micelle surface and the thermal motion of the chains is bound, in the course of time, to bring all the segments into contact, if only briefly, with the water. Similar arguments can be applied to the phospholipid bilayer above its phase-transition temperature. Under these conditions the area per phospholipid molecule is about 70 \AA^2 (131). Since the polar group occupies only about 40 \AA^2 , some 30 \AA^2 of hydrocarbon per molecule must be exposed to the water. This is equivalent to three or four methylene groups.

The detection of water within the hydrocarbon region of the bilayer by ESR spectroscopy appears at first sight an attractive proposition. However, spectroscopic probes, such as those employed in the ESR technique, are exceptionally unsuitable for the detection of water in micelles and bilayers, because the probes must have considerably greater affinities for water than the hydrocarbon moieties normally present.

Within bilayers at temperatures above their transition temperatures, the order parameter for the various carbon atoms decreases along the chain, reaching a minimum at the terminal methyl groups (125, 126). More relevant to the problem of water permeability is the deduction of D. Gruen (personal communication) that in a liquid bilayer the chains are sufficiently delocalized that the order at various points across the hydrocarbon region varies remarkably little. This conclusion does not hold when cholesterol is present. In such instances the segments adjacent to the rigid cholesterol ring system are always restricted in their motion and the average order in the bilayer now decreases appreciably toward the center (133).

If the rate-determining step for the movement of water across the bilayer is the diffusion through the hydrocarbon-chain region, a knowledge of the segmental motions of the lipids is likely to be necessary for a detailed understanding of the process. Unfortunately it is by no means clear either how the water is distributed or how it moves. Various estimates have been made of the "microviscosity" of the bilayer (8, 36, 38, 128). These are useful if it is desired to produce a continuum model for the water transfer, but the microviscosities differ according to the manner of their determination and it is not obvious which values are the most relevant to the present problem.

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ntal-Mueller "solventless" (1). For this capacity to give diffraction it is necessary to fit for *n*-alkanes, i.e., about aken to indicate that water has been shown, however, that the value is about $0.74 \mu\text{F cm}^{-2}$, consistent with the X-ray data

(7, 28, 95, 96) shows that all segments exposed to water, but it is a considerable amount of the thermal motion of the segments into contact, if it can be applied to the temperature. Under these about 70 \AA^2 (131). Since the hydrocarbon per molecule of three or four methylene

region of the bilayer by ESR is proposition. However, the ESR technique, are in micelles and bilayers, affinities for water than the

transition temperatures, the increases along the chain, (25, 126). More relevant to on of D. Gruen (personal sufficiently delocalized that region varies remarkably when it is present. In such a sol ring system are always the bilayer now decreases

water across the bilayer is on, a knowledge of the necessary for a detailed no means clear either how imates have been made of se are useful if it is desired , but the microviscosities and it is not obvious which

E. Relation Between Osmotic Flow and Tracer Diffusion

Experiments on biological membranes have usually yielded values for the diffusional permeability P_d that were smaller than the osmotic permeability P_t , with P_t/P_d depending on the type of membrane (72, 106). Aside from any differences caused by unstirred layers, the relation between the two permeability measurements has been considered of interest because it was thought to be associated with the mechanism by which water crosses the membrane.

For unmodified lipid membranes where the concentration of water in the membrane is thought to be extremely small there should be no interactions between the water molecules. Hence the values for P_t and P_d should be equal and this is the experimental finding if corrections are made for the unstirred layers. If water moves through aqueous pores, however, P_t can be larger than P_d , as has been demonstrated for lipid membranes rendered porous with gramicidin or one of the polyene antibiotics. For large pores this inequality could arise because under an osmotic pressure gradient the water moves by viscous flow, whereas the labeled water molecules move simply by diffusion (80, 97, 102). Assuming Poiseuille flow for the net water movement, the ratio P_t/P_d can be related to r , the radius of the pore, by

$$r^2 = \frac{8D_w \eta_w \bar{V}_w}{RT} [(P_t/P_d) - 1] \quad (19)$$

where D_w and η_w are the diffusion coefficient and viscosity of the water in the pore. For example, consider a pore the size of that created by nystatin ($r \approx 4 \text{ \AA}$): with bulk values for D_w and η_w , Equation 19 predicts that the pore radius should be about 5 \AA for the experimental P_t/P_d ratio of about 3.0 (71).¹ To some extent, this agreement may be fortuitous and may be due to several errors canceling. For the gramicidin pore, where $r \approx 2 \text{ \AA}$, the predicted P_t/P_d with Equation 19 is 1.3, which is substantially different from the experimental value of 5.3 (120).

The validity of a calculation of the pore radius based on the continuum hydrodynamic theory has been checked experimentally for pores several tens of angstroms in radius (14). Although the calculation may still give tolerable answers even for pores a few angstroms in radius (83), the validity of the theory must become questionable when r is comparable to the dimensions of the water molecule; in addition it is probably invalid to assign bulk values for D_w and η_w for the inside of the pore. Alternative theoretical treatments have been applied

¹ A correction sometimes introduced (97) when r becomes comparable to a_w , the radius of the water molecule, is to replace r^2 in Equation 19 by λ , where r and λ are related by

$$r = (2a_w^2 + \lambda)^{1/2} - a_w$$

If this correction is used when calculating the radius of the nystatin pore from P_t/P_d , the agreement with the experimental value is even better.

when the pore becomes sufficiently narrow that only a single file of water molecules can be accommodated along the length of the pore and solute and solvent molecules cannot overtake. As an extension of the single-file kinetics (65, 66, 68, 70) and explicit in other approaches (35, 82, 84) is the prediction that P_f/P_d will have a value near n , the average number of water molecules in the pore; clearly n can be larger than unity. One other theoretical treatment has indicated that P_f and P_d should be equal under single-file conditions (91). The pores created by gramicidin are sufficiently narrow ($r \approx 2 \text{ \AA}$) that the movement of water molecules through them probably approximates to single filing. The P_f/P_d ratio of 5.3 measured for gramicidin (120) suggests that the latter theoretical analysis is inapplicable. Although this review is not concerned with the detailed properties of the gramicidin pore, it should be noted that the average number of water molecules in the pore has been inferred to be 6 (119) or 12 (85). These values have been deduced from measurements of streaming potentials produced by osmotic pressure differences across gramicidin-containing membranes and also from the electro-osmotic flux per ion.

One interesting conclusion that can be drawn is that there is not necessarily a monotonic relationship between the P_f/P_d ratio and the pore radius. This makes it impossible to determine the pore dimensions solely from the measurements of osmotic and diffusional permeabilities.

F. Water Permeability of a Biological Membrane

In this final section the general properties of water transport in artificial membranes are compared with those found for the plasma membrane of the erythrocyte. The techniques used to measure the water permeability of liposomes (see sect. III) have also been previously applied to the erythrocyte (31, 51, 97, 130). For the cell membrane the techniques might be expected to give more accurate answers, because each cell in a suspension is uniform and has a single membrane of known area. The lipid composition of the membrane in mammals consists of approximately an equimolar mixture of cholesterol and phospholipid, with the phospholipids being sphingomyelin, phosphatidyl-choline, phosphatidylethanolamine, and phosphatidylserine (122).

The osmotic permeability of erythrocytes of various mammals has been measured and in almost all cases the value of P_f has been greater than $100 \mu\text{m/s}$ at room temperature (51). For the human erythrocyte the osmotic and diffusional permeabilities are given in Table 6. These permeability values and the P_f/P_d ratio are all larger than one would expect for comparable artificial membranes. Thus, for example, artificial membranes made with the phospholipids extracted from sheep HK erythrocytes plus cholesterol were found to have a $P_f = 16.8 \mu\text{m/s}$ and $P_d = 13.8 \mu\text{m/s}$ ($P_f/P_d = 1.22$) at 26.5°C (5). These membranes probably did not contain equimolar phospholipid and cholesterol; if they had, these permeabilities could have been even lower. By comparison a membrane containing an equimolar mixture of egg phosphatidylcholine and

by a single file of water molecules in the pore and solute and the single-file kinetics (65, 66), the prediction that P_r/P_d is 1 for molecules in the pore; this treatment has indicated conditions (91). The pores (10 Å) that the movement of water molecules is due to single filing. The P_r/P_d ratio is 1, which is consistent with the latter theoretical prediction. Concerned with the detailed mechanism of water transport, it is found that the average number of pores is 9 (89) or 12 (85). These values are consistent with the water potentials produced by various membranes and also

at there is not necessarily a single pore radius. This is consistent with the predictions solely from the theory.

Water transport in artificial membranes is similar to that in the plasma membrane of the erythrocyte (31, 32). The water permeability of the erythrocyte (31, 32) might be expected to give a similar result. The diffusion is uniform and has a single pore radius. The composition of the membrane is a mixture of cholesterol and phosphatidylserine (122).

Water permeability in various mammalian erythrocytes has been found to be greater than 100 $\mu\text{m/s}$ (33). In the erythrocyte the osmotic and water permeability values are similar. For comparable artificial membranes made with the phosphatidylserine and cholesterol were found to be 1.22 at 26.5°C (5). These values are lower than those for phospholipid and cholesterol; if the phospholipid is replaced by cholesterol, the water permeability is even lower. By comparison a membrane made with phosphatidylcholine and

cholesterol might be expected to have a P_r and P_d of 10–15 $\mu\text{m/s}$ at the same temperature. (The chain composition of egg phosphatidylcholine is roughly similar to the erythrocyte phospholipids.) The osmotic permeabilities of comparable artificial membranes are thus about an order of magnitude smaller than the values for the erythrocyte. This suggests that the additional permeability is due to aqueous pores in the erythrocyte membrane, and is consistent with this idea is the low activation energy for transport and also the high P_r/P_d ratio. This ratio is not accounted for by unstirred layers, as these have been estimated to be only about 5 μm in a permeability experiment (127) and are therefore negligible.

An argument that seems to confirm this conclusion about the presence of pores is the fact that after treatment of human red cells with *p*-chloromercuribenzosulfonic acid, the water permeability was reduced to that expected for the equivalent artificial membrane (89, 90). The osmotic permeability decreased from 200 $\mu\text{m/s}$ to 20 $\mu\text{m/s}$, the ratio of osmotic to diffusional permeability became close to unity, and the activation energy for water transport increased from 4 to 11.5 kcal/mol (90). The erythrocytes of the chicken, unlike mammals, have water permeability properties closer to those of artificial membranes and *p*-chloromercuribenzosulfonic acid-treated human red cells (Table 6). This may indicate that they do not contain such pores.

One curious feature about these "pores" in the human erythrocyte is that although they may contribute a large additional water permeability they add very little ion permeability. Thus the conductance of the membrane of the human erythrocytes has been estimated to be about $4 \times 10^{-6} \Omega^{-1} \text{ cm}^{-2}$ at 25°C (75). Even if all this conductance were contributed by the pores, which is not necessarily the case, then the pores would have a water permeability of about $5 \times 10^5 \mu\text{m/s}$ normalized to a membrane conductance of $10^{-2} \Omega^{-1} \text{ cm}^{-2}$. This is much larger than the comparable values for gramicidin or nystatin (see sect.

TABLE 6. Water permeabilities of erythrocytes at room temperature (19–25°C)

System	Permeability, $\mu\text{m/s}$			E_a , kcal/mol	Ref.
	P_r	P_d	P_r/P_d		
Human erythrocytes*	173	53	3.3	3.9	97, 118, 140
Human erythrocytes + 1 mM <i>p</i> -chloromercuribenzosulfonic acid	20	18	1.1	11.5	90
Chicken erythrocytes	21†	13.5	1.5	11.4	19, 21, 43

Activation energy (E_a) is that for the osmotic permeability. * Values given here are typical, but more extensive data are summarized in review by Forster (51). † This value is the mean from 2 sets of measurements (19, 43).

II_E), and indicates that the water-to-ion selectivity for the erythrocyte pore is very high.

The permeabilities for mammalian erythrocytes are toward the upper end of the range of water permeabilities for biological membranes (72). By comparison, egg cells that have to survive in freshwater have water permeabilities of 1 $\mu\text{m/s}$ or less (81, 106), which is toward the lower end of the biological range. It is not clear how such low permeabilities are achieved. From results with artificial membranes the presence of sphingolipids with long and saturated chains would be expected to produce water permeabilities of the right order of magnitude, but at present no data are available on the lipid composition of the frog or toad egg cell membrane to confirm this as the mechanism.

V. SUMMARY

Knowledge of the permeability of lipid bilayers to water has been gleaned largely from studies with black-lipid films, on the one hand, and multi- and single-walled lipid vesicles, on the other. These two experimental systems, the techniques required for their study, the results they yield, and the relevance of these results to those expected for the type of lipid structure thought to be present in biological cell membranes form the main part of this review. The mechanisms by which water may cross the bilayer are considered in the light of recent spectroscopic and other evidence about the structure of the bilayer. The modification of lipid membrane permeability brought about by the incorporation of pore-forming molecules is discussed briefly.

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